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(54) **GLYCOPROTEIN OF HUMAN ORIGIN, PHYSIOLOGICALLY ACTIVE FACTOR COMPRISING THE SAME,  
AND PHARMACEUTICAL PREPARATION CONTAINING THE SAME AS ACTIVE INGREDIENT.**

(57) A glycoprotein is obtained from a supernatant of a culture medium of the fibroblasts of human origin. It has an antitumor activity and activities of inducing the differentiation of leukemia cells, enhancing cell-mediated immunity, accelerating the proliferation of endothelial cells of human blood vessel, and proliferating hepatic parenchymal cells.

**EP 0 462 277 A1**

Field of the Invention

The present invention relates to a glycoprotein obtained from the culture broth of the human-derived fibroblasts, biologically active factor which includes glycoprotein, and pharmaceutical product which comprises biologically active factor as active component.

The glycoprotein in the present invention, which shows cytotoxic activity to various tumor cell lines but not to normal cells, is a new tumor cytotoxic factor, leukemic cell differentiation inducing factor, cellular immunology enhancing factor, vascular endothelial cell growth factor and hepatocyte growth factor. This material is useful as an anti-tumor drug, an anti-leukemia drug, a cellular immunology enhancing drug, a wound healing drug and a liver regenerating drug, etc. or a biochemical or pharmacological reagent.

Description of the Related Art

$\beta$ -Interferon is a representative factor as the biologically active factor, for example, the tumor cytotoxic factor which is produced by human-derived fibroblasts. This is a glycoprotein which is secreted by the fibroblasts, when after the culture, the cells are harvested and stimulated by poly I-polyC or sendai viruses. It has been clarified that the protein has various physiological activities in addition to its anti-virus or anti-tumor action. A fibroblast-derived tumor cytotoxic glycoprotein called as CBF is represented in Japanese Laid-Open No. 58-146293. A tumor growth inhibitory factor (INF) with a molecular weight of 35,000 to 45,000 which is purified from the culture broth of human tissue-derived fibroblasts is disclosed in Japanese Laid Open No. 671-33120. Also, a tumor necrosis factor-like material which is purified from the culture broth of fibroblasts, a fibroblast-derived necrosis factor, FNF, and a biologically active material with the cytotoxic activity, which is produced by animal fibroblast cells and has a molecular weight of 40,000 to 50,000 and an isoelectric point of  $5.0 \pm 0.5$ , are disclosed in Japanese Laid Open No. 61-56131, Japanese Laid Open No. 61-1872, and Japanese Laid Open No. 62-103021, respectively. Furthermore, all amino acid sequence and cDNA sequence coding for the amino acid sequence of a tumor cytotoxic factor, which is obtained from the culture broth of human-derived fibroblasts, with a molecular weight of  $36,000 \pm 1,000$  and an isoelectric point more than 10.5, are disclosed in Japanese Laid Open No. 64-10998.

Summary of the Invention

The present inventors have investigated about a biologically active material which is contained in the culture broth of human-derived fibroblasts and have found a glycoprotein with various biological activities, which is different from the materials reported previously in points of the molecular weight and the isoelectric point etc.

Therefore, the main aim of the present invention is to offer the new glycoprotein, biologically active factor which includes said glycoprotein, and pharmaceutical product which comprises said biologically active factor.

A new human fibroblast-derived glycoprotein in the present invention (it is called as TCF-II hereafter) is characterized by the following physicochemical properties.

- a. Molecular weight; On SDS gel electrophoresis,  $78,000 \pm 2,000$  or  $74,000 \pm 2,000$  under the nonreduced conditions and a common band A with  $52,000 \pm 2,000$  and band B with  $30,000 \pm 2,000$  or band C with  $26,000 \pm 2,000$  under the reduced conditions.
- b. Isoelectric point; 7.4 to 8.6.
- c. Heat stability; Stable in the heating at  $60^\circ \text{C}$  for 10min.
- d. pH stability; Stable in the range of pH6 to 9.
- e. Carbohydrate chain; Adsorbed to a Concanavalin A (Con A)-Sepharose column.
- f. Biological activity; Inhibit the growth of KB cells, HeLa cells, and L-929 cells but not IMR-90 cells.
- g. Reactivity to antibodies; The cytotoxic activity is not neutralized by anti-TNF antibody, anti-lymphotoxin antibody, and anti-interferon- $\beta$  antibody.

Furthermore, the suitable lot of TCF-II in the present invention has the following properties in addition to the above physicochemical characteristics (a~g):

- h. N-terminal amino acid sequence; The above mentioned band B and band C are subchains of band A, respectively. N-terminus of band A is blocked. Band B and band C has common N-terminal amino acid sequence as follows; Val-Val-Asn-Gly-Ile-Pro-Thr-  
or

Val-Val-Asn-Gly-Ile-Pro-Thr-X-Thr-Asn-Ile-Gly-X-Met-  
 Val-Ser-Leu-

X means an unidentified amino acid.

i. Amino acid composition; When it is hydrolyzed with HCl, it exhibits the following amino acid composition.

10

	A.A	nmol	mol%
15	Asp	10.375	12.97
	Glu	7.750	9.69
	Ser	5.000	6.25
20	Gly	7.250	9.06
	His	3.000	3.75
25	Arg	5.375	6.72
	Thr	5.125	6.41
	Ala	2.625	3.28
30	Pro	5.625	7.03
	Tyr	3.875	4.84
35	Val	4.125	5.16
	Met	1.875	2.34
	Cys	ND	-
40	Ile	5.000	6.25
	Leu	4.875	6.09
45	Phe	2.250	2.81
	Trp	ND	-
	Lys	5.875	7.34
50			
	total	80.000	100(99.99)

55 Furthermore, all the primary sequence of TCF-II in the present invention was deduced from its complementary DNA (cDNA). TCF-II cDNA was cloned by screening cDNA library which was prepared by using mRNA purified from total RNA which was extracted from human embryonic fibroblast cells (IMR-90), according to the following method.

(1) Extraction of poly(A)<sup>+</sup> RNA from IMR-90 cells

Total RNA was prepared by guanidine thiocyanate-cesium chloride method (Biochemistry 18 5294-5299(1979)) from  $2 \times 10^8$  IMR-90 cells which were cultured in the Dulbecco's modified eagle (DME) medium containing 5% of new born calf serum (NBCS). The IMR-90 cells were suspended in 28ml of 6M guanidine thiocyanate containing 5mM sodium citrate, 0.5% Sarcoseal and 0.1M  $\beta$ -mercaptoethanol, and were homogenized. 5.7M cesium chloride solution, 4ml containing 0.1M EDTA was put into polyallomer centrifuge tubes. The homogenized solution, 7ml was loaded on the cesium chloride solution and then centrifuged at 35,000 rpm, 20 ° C for 16 hours, using 40TI rotor of Beckman centrifugator. After centrifugation, the pellets were washed twice with 95% ethanol and dissolved in 200  $\mu$ l of 10mM Tris HCl buffer (pH7.5) solution containing 1mM EDTA by heating at 65 ° C for 5 min, designated as total RNA solution. Poly (A)<sup>+</sup> RNA was purified from the total RNA by the method of oligo (dT)cellulose-column chromatography. The total RNA solution was loaded on the oligo (dT) cellulose-column which was equilibrated with 10mM Tris HCl buffer(pH7.4) solution containing 1mM EDTA, 0.5M NaCl and 0.05% SDS. The adsorbed fraction was eluted with 10mM Tris HCl buffer, pH7.4, containing 1mM EDTA and 0.05% SDS , and designated as poly(A)<sup>+</sup> RNA solution.

## (2) Synthesis of cDNA library

Double strand cDNA was synthesized by using poly (A)<sup>+</sup> RNA from (1) as a template and by using cDNA synthesis kit (Pharmacia Co. Ltd), and was attached EcoR I adaptor. The method of synthesis was performed according to the protocol of Pharmacia Co.Ltd, except addition of reverse transcriptase (40 units/reaction mixture, Life Science Co. Ltd ) derived from non-sphere disease virus of avian bone marrow at the synthesis of single strand DNA.

## (3) Preparation of cDNA library

The cDNA obtained from (2) was inserted in EcoR I arm (Promega Co. Ltd) of phage vector  $\lambda$  gt10. 3.3  $\mu$ g of cDNA synthesized from poly(A)<sup>+</sup> RNA was dissolved in 150  $\mu$ l of column buffer, 66 mM Tris-HCl buffer (pH7.6) containing 1mM spermidine, 10mM magnesium chloride, 15 mM dithiothreitol and bovine serum albumin (0.2 mg/ml). 5.2  $\mu$ l of the above solution was mixed with 1  $\mu$ g of  $\lambda$ gt 10 EcoR I arm, and then precipitated with ethanol. Recombinant phage DNA including both  $\lambda$ gt 10 and cDNA was prepared as follows. The above precipitate was reconstituted in 9  $\mu$ l of the column buffer and was incubated at 16 ° C overnight by adding 1  $\mu$ l of 10mM adenosine triphosphate and 1  $\mu$ l of T4 DNA ligase (350 units/ $\mu$ l).

## (4) Screening of cDNA library

## (i) Preparation of oligonucleotide probe

For preparation of probe, a mixture of complementary oligonucleotide of 17 mer (384 species mix) corresponding to the amino acid sequence from Val<sup>1</sup> to Pro<sup>6</sup> in N-terminal amino acid sequence of TCF-II  $\beta$ -chain was synthesized and labelled 5' terminus with T4 polynucleotide kinase (TAKARA SHUZO) and [ $\gamma$ -<sup>32</sup>P]ATP (Amersham, Co, Ltd). This probe is shown as following;  
complementary strand used as probe:

(384 species mix )

3' -CACCACCTTACCGTAGGG- 5'

G	G	G	C	A
A	A		A	T
T	T		T	

(ii) screening of recombinant phage

About 500 thousand plaques of phage were obtained by in vitro packaging of the recombinant phage DNA solution which was obtained by (3), using Gigapack Gold (Stratagene) and then by infecting to E.Coli C600hfl. After adsorption of the plaques to Hybond-N filter (Amersham), they were denatured with alkali, neutralized and baked at 80° C for 2 hours. Hybridization was performed by the method of Bell et al (Nature 310 775 -777, 1984). The first screening was carried out by using the mix probe which was obtained from (1). One clone which would contain TCF-II fragment was found in the positive plaques detected by the first screening.

(5)Cloning of full length of TCF-II cDNA

Internal amino acid sequences (one letter code), ( $\alpha$ ) NYMGNLSQTRSGL and ( $\beta$ ) TSXSVYGWGYTGILNYDGLL(X: not identified) were obtained respectively from  $\alpha$ - and  $\beta$ -chains of TCF-II by digesting them with lysylendopeptidase and subsequently by mapping their fragments. N-terminal amino acid sequence of  $\beta$ -chain of TCF-II was coincided with that of  $\beta$ -chain of one of the human hepatocyte growth factors (hHGFs). Moreover, the above mentioned internal amino acid sequences ( $\alpha$ ) and ( $\beta$ ) in TCF-II were located in  $\alpha$ - and  $\beta$ -chain of both hHGF, respectively. Therefore, it has been thought that TCF-II is expressed from one of the family of hHGF genes. MIYAAWA et al, (BBRC 163 ,967-973 (1989)) and NAKAMURA et al. ( Nature. 342 ,440-443 (1989)) reported the hHGF cDNAs from placenta and liver cDNA libraries, respectively.

Comparison of all the primary sequence deduced from both hHGF cDNAs revealed differences in amino acids at 14 sites in their sequences. From these results, the presence of the family of hHGF genes was suggested. Identical regions between placenta type and liver type hHGF cDNAs were chosen as primer sequences for Polymerase Chain Reaction (PCR). The identical oligonucleotides of both hHGF cDNAs at 5' and 3' non-coding region were chemically synthesized and screening of TCF-II cDNA was carried out by PCR using them as primers. Sal-77 primer which have a cleavage site of restriction enzyme Sal I and Sph2203 primer which have a cleavage site of restriction enzyme Sph I were synthesized by DNA synthesizer (Applied Co. Ltd). These primers are shown as following:

Sal-77 primer: 5'-GGTCGACTAGGCACTGACTCCGAACAGGATTC-3'

Sal I

Sph2203 primer: 5'-GGCATGCACAGTTGTATTGGTGGGTGCTTCAG-3'

Sph I

cloning with PCR method was carried out by following procedures,

(i)PCR

	cDNA synthesized as described in (2)	1 $\mu$ l
	(dissolved in 150 $\mu$ l of column buffer)	
5	20 $\mu$ M Sal-77 primer	2.5 $\mu$ l
	20 $\mu$ M Sph2203 primer	2.5 $\mu$ l
10	10 $\times$ PCR reaction solution	10 $\mu$ l
	(100mM Tris HCl buffer (pH8.3) containing 500mM KCl, 15 mM MgCl <sub>2</sub> and 0.1% (W/V) gelatin)	
15	1.25mM dGTP, dATP, dTTP, dCTP, mixture	16 $\mu$ l
	Ampli Taq (5 units/ $\mu$ l TAKARASHUZO)	0.5 $\mu$ l
20	Distilled water	67.5 $\mu$ l

After above solutions were mixed in microfuge tube with 0.5 ml volume size and covered on the liquid surface with 100  $\mu$ l of mineral oil (Sigma Co. Ltd). PCR was carried out by the Quick Thermo System (Japan Genetics Co. Ltd). After pretreatment at 94 °C for 7 min, a three-step reaction which consists of annealing reaction, at 55 °C for 3 min; polymerase reaction, at 72 °C for 2min; and denature reaction, at 94 °C for 2 min was repeated 35 times. Then the reaction mixture was treated for 3 min at 55 °C, and subsequently for 11min at 72 °C and then returned to room temperature (Each time includes the altering time of temperature). When a part of the reaction mixture was analyzed an electrophoresis using agarose gel, a DNA fragment consisting of about 2.3 kilobases (Kb) which was thought as aimed TCF-II cDNA, was obtained. Then, the DNA which was obtained from four tubes containing the above mentioned reaction mixture was precipitated with ethanol and digested with restriction enzymes, Sal I and Sph I. After an agarose gel electrophoresis, DNA fragment with about 2.3Kb was recovered by using DE81 paper (Watman Co. Ltd).

#### (ii) Subcloning

The DNA fragment with 2.3Kb digested with restriction enzymes, Sal I and Sph I, which was obtained from (1), was inserted using ligation kit (TAKARA SHUZO) into a vector fragment which was obtained by digestion of plasmid vector pUC18 (Japan gene Co. Ltd) with restriction enzymes, Sal I and Sph I, and transfected into Esherichia Coli DH5 $\alpha$  (according to protocol of BRL Co. Ltd). More than 20 subclones could be obtained.

#### (iii) Determination of base sequence

The base sequences of obtained subclones were determined by the dideoxy-method (Sequeanase Ver. 2.0 TOYOBO). Incorporation errors of nucleotides on the AmpI Taq (TAKARA SHUZO) were corrected by the analysis of base sequences of several subclones. The base sequence of TCF-II cDNA obtained by the above mentioned procedure and amino acid sequence deduced from the base sequence are shown in Fig.15. It consists of 2172 base pairs (bp) from ATG of initiation codon for transcription to TAG of termination codon. If translated into amino acid, TCF-II consists of 723 amino acids. Amino acid sequence from the first methionine (Met<sup>1</sup>) to the 29 th alanine (Ala<sup>29</sup>) residues is presumed as a signal sequence. As shown in Fig.15, TCF-II in which two polypeptides consisting of  $\alpha$  chain and  $\beta$  chain are bound by disulfide bond is synthesized initially as a single chain. Since N-terminus of  $\alpha$  chain in TCF-II was blocked, it was unidentified. However, N-terminal amino acid sequence of  $\beta$  chain and a few internal amino acid sequences of TCF-II were determined as above mentioned, and were shown in Fig.15.

The obtained base sequence of the TCF-II cDNA is very similar to hHGF which has been found by MIYAZAWA et al (Biochemical and Biophysical Research Communication 163 967-973 (1989)). TCF-II cDNA,

however, deletes five amino acid residues (F-L-P-S-S) from Phe<sup>162</sup> to Ser<sup>166</sup> in the amino acid sequence of hHGF. Therefore, the facts revealed that TCF-II cDNA was new one of the family of hHGF genes. The comparison between the amino acid sequence of TCF-II, which is deduced from above mentioned base sequence, and the amino acid sequence of hHGF (MIYAZAWA et al) is shown in Fig. 16.

5 The method to obtain the new glycoprotein, TCF-II which is characterized by the above mentioned physicochemical properties is described in the followings.

Any human-derived fibroblast can be used as a cell for the production of the material in the present invention. Human embryonic lung-derived fibroblast cells, human embryonic kidney-derived fibroblast cells, and human embryonic prepuce-derived fibroblast cells etc. are given as suitable cells. In the performance of  
10 the present invention, IMR-90 cells (ATCC CCL 186) and WI-38 cells (ATCC CCL 75) etc. are preferably suitable.

These cells are grown in serum-enriched medium or serum-free medium which are used in the common cultures. Dulbecco's modified Eagle medium (DMEM) containing 5% bovine calf serum is given as the representative medium. Amino acids, transferin, fatty acids and hormones such as insulin etc. can be  
15 added if necessary.

The cells are cultured in the medium, and the standing culture using T flask etc., the floating culture using microcarrier, and the continuous culture using hollow fiber or ceramic carrier are able to be adopted. It is preferable that the culture is carried out in atmosphere with 5% CO<sub>2</sub> at 20 to 37°C as the culture conditions and that the medium is exchanged every 2 to 3 days. After the cell density reaches the  
20 optimum, the medium is exchanged every 7 to 10 days and the culture broth is collected. The aimed glycoprotein is purified from the collected culture broth.

The collected culture broth is concentrated by UF concentration using a membrane with a pore size of M.W. 6,000. The aimed glycoprotein in the UF concentrate is adsorbed to cation exchange resins and then eluted from the resins with buffer containing 0.3 ~ 0.6M NaCl. CM Sephadex (Pharmacia) C-50 etc. can be  
25 given as the ion exchange resins. The fractions which have the potent cytotoxic activity are collected and subsequently applied to affinity chromatography for glycoprotein. ConA-Sepharose is especially suitable to affinity chromatography for the aimed glycoprotein. The affinity column is equilibrated with 0.05M Tris-HCl buffer, pH7.0, containing 0.5M NaCl, and then the above obtained fraction is applied to the column. After washing the column with the equilibration buffer, the active material is eluted from the column with an elution  
30 buffer containing carbohydrate corresponding to carbohydrate chain attached to the affinity column (or gel) for glycoprotein. When the above mentioned ConA-Sepharose is used, the active material is eluted with the buffer containing  $\alpha$ -methyl-D-mannopyranoside. The eluted active fraction is dialysed against water and lyophilized. The lyophilized active material is dissolved with 0.05M Tris-HCl buffer, pH6.0 to 7.0, containing 0.2M NaCl and is further purified on HPLC using a strong cation exchange column. Mono S (Pharmacia) is  
35 especially suitable to the strong cation exchange column. Elution of the active material from the Mono S column is carried out with a gradient from 0 to 1.0M NaCl and the active fractions are collected.

The present active material is eluted at salt intensity of 0.6 to 0.9M. Thus obtained active fraction is further purified on an affinity chromatography using Heparin-Sepharose (Pharmacia). Elution of the active material from the Heparin-Sepharose column is carried out by a gradient from 0.3 to 2.0M NaCl and the  
40 aimed material is eluted at salt intensity of 1.0 to 1.5 M. Subsequently, assays for cytotoxic activity of the present inventional material, TCF-II to mouse L929-18 cells and for growth stimulating activity of TCF-II for hepatocytes are mentioned below.

#### Assay for Cytotoxic Activity

45 Mouse L929 cells (ATCC CCL1) were subcloned and a subclone with the highest sensitivity to TCF-II in the present invention was selected. Thus, the clone, L-929-18 which had a high sensitivity to the tumor cell cytotoxic factor was obtained.

L-929-18 cells were grown to confluence in DMEM containing 10% FCS, and then the cells were  
50 harvested by trypsin treatment. The cells were suspended at a cell density of  $6 \times 10^5$  cells/ml in DMEM containing 10% FCS and 1  $\mu$ g/ml actinomycin D. 50  $\mu$ l of DMEM which was prepared in the same way as the cell suspension was added to each well in 96-well microplate (Falcon) and 50  $\mu$ l of the sample solution which contains the present substance, TCF-II, dissolved in the same DMEM was added to the first dilution well. Both were mixed well and 50  $\mu$ l of the mixture was subsequently added to the second dilution well. A  
55 serially diluted substance was prepared by repeating the above procedures.

50  $\mu$ l of the cell suspension was inoculated into each well containing a serially diluted substance and the culture was carried out at 37°C for 2 days in a CO<sub>2</sub> incubator. After the culture, the medium was removed gently and the cells were washed twice with saline. The viable cells which adhere to each well

were fixed and stained by addition of 50  $\mu$ l of 0.5% crystal violet in the mixture of methanol and water (1:4) to each well. Each well was washed three times with distilled water and dried, and the crystal violet in each well was extracted with Sorenson's buffer (mixture of 6.1 ml of 0.1 M disodium citrate, 3.9 ml of 0.1N HCl, and 10 ml of ethanol). Absorbance of the extracts at 570 nm was determined by a microtiter spectrophotometer.

Unit of TCF-II (u/ml) was defined as the dilution ratio given 50% cell death.

#### Assay for hepatocyte growth stimulating activity

Hepatocytes were separated from Wister male rat by the method of Segren (Method in cell biology, vol. 13, p29, Academic Press, New York, 1976). The obtained hepatocytes were planted on 24 well plastic plates (Falcon) at the cell density of  $8.8 \times 10^4$  cells/0.5ml/well and cultured under the presence of 5% CO<sub>2</sub> at 37°C. A Williams E culture medium (Flow Raboratory) which was supplemented with 10% fetal bovine serum (Hyclone), 100 u/ml penicillin and 100  $\mu$ g/ml streptomycin was used as the culture medium (abbreviated below as basal culture medium). After incubation at 37°C for 24 hours, the culture medium was exchanged with the basal culture medium containing the test samples. The hepatocytes were further cultured for 24 hours and then cultured in the basal culture medium containing 4  $\mu$ Ci/ml (86 Ci/m mol) of <sup>3</sup>H- thymidine (Amersham) for 2 hours followed by determination of DNA synthesis. When the hepatocytes were labeled with <sup>3</sup>H-thymidine, the incorporated counts (dpm) were determined from the difference of counts(dpm) which were measured between the presence and absence of 10 mM hydroxyurea on each test groups. After the cells were labeled by the above procedure, the cells were washed twice with cold PBS, 2% perchloric acid and 95% ethanol and dried by air, then dissolved in 0.8 ml of 2% SDS containing 2mM EDTA and 20mM NaHCO<sub>3</sub> followed by determination with a liquid scintillation counter.

The result is shown in Table 1.

Table 1

Sample	Concentration (ng/ml)	Hepatocyte growth stimulating activity (dpm/well, $\times 10^3$ )
No addition	-	21.7 $\pm$ 9.2
hEGF	20	239.3 $\pm$ 7.2
TCF- II	1	93.7 $\pm$ 29.7
	10	378.5 $\pm$ 93.5
	100	467.4 $\pm$ 77.3

hEGF (WAKUNAGA Pharmacy Co Ltd) was used as the positive control for hepatocyte growth stimulating activity. The results in Table 1 demonstrate that hepatocyte growth stimulating activity of TCF-II is stronger than that of hEGF.

Nextly, the present invention describes pharmaceutical preparations which contains biologically active



factor, TCF-II, as an active component.

The above mentioned biologically active factor in the present invention has following pharmaceutical activities:

① An antitumor activity:

5 TCF-II inhibits the growth of KB, HeLa, MCF-7 and BG-1 which is human-derived tumor cells, and have a cytotoxic activity to mouse L-929 cells, and to Sarcoma 180, Meth A Sarcoma and P388 of tumor cells, but not inhibit the growth of IMR-90 cells which are human-normal cells.

② A leukemia cell differentiation inducing activity:

TCF-II induces the differentiation of the human-derived leukemia cell line, HL-60 to granulocyte like cells.

10 ③ A cellular immunology enhancing activity:

TCF-II stimulates the growth of human cytotoxic T cells

④ A vascular endothelial cell growth stimulating activity:

TCF-II stimulates the growth of human umbilical cord blood vessel-derived endothelial cells.

⑤ A hepatocyte growth stimulating activity:

15 TCF-II stimulates the growth of adult rat hepatocytes.

These activities are exhibited in very small dose range of 1-1000 ng/ml.

The pharmaceutical product in the present invention can be expected as an anti-tumor drug, an anti-leukemia drug, a cellular immunology enhancing drug, wound healing drug and therapeutic drug for liver disease including liver regenerating drug and so on. The biologically active factor (it is called as TCF-II hereafter) which is a glycoprotein with high molecular weight, however, is easily adsorbed to a polypropylene vessel and so on, which is an injection syringe, and to a glass vessel and so on.

TCF-II is also an unstable substance. The activity of TCF-II is considerably decreased by temperature or humidity and so on, and inactivated easily. Therefore, manufacturing of the stable pharmaceutical product is required. Accordingly, the pharmaceutical product in the present invention is a TCF-II one which contains 25 one or more of proteins and nonionic detergents as adsorption preventors, or one or more of proteins, carbohydrates and amino acids as stabilizing agents, or moreover the combination of one or more as adsorption preventors and one or more as stabilizing agents.

Any pharmaceutical form which is a lyophilized, or liquid or powder form can be used as a pharmaceutical product in the present invention, if the above mentioned adsorption preventors and 30 stabilizing agents were included in the pharmaceutical product. The active component in the present invention, TCF-II can be produced or purified by the any method. TCF-II which is purified from culture broth of any TCF-II secreting cell, can be also used. TCF-II which is produced by the method of recombinant DNA technology, using Escherichia Coli, yeast, mammalian cells such as chinese hamster ovary etc, as hosts, and is purified by various methods, can be also used.

35 Albumin and gelatin etc, among proteins, and Tween 80 and Tween 20 etc, among nonionic detergents can be used as the adsorption preventor in the present invention.

Albumin and gelatin etc, among proteins; sorbitol, mannitol and xylitol etc, among carbohydrates; glycine and alanine etc, among amino acids can be used as the stabilizing agents in the present invention. The suitable added amounts of proteins as the adsorption preventors are more than 0.1%, preferably 0.1 to 40 20%, and the suitable added amounts of nonionic detergents are more than 0.001%, preferably 0.001 to 1.0%. The suitable added amounts of proteins as stabilizing agents are more than 0.1%, preferably 0.1 to 20.0%; the suitable added amounts of carbohydrates are 5 to 40%; and the suitable added amounts of amino acids are more than 1%. On the case of using with combination of one or more of adsorption preventors and one or more of stabilizing reagents, or using with combination of two or more of stabilizing 45 agents, the added amounts of each agent were permitted in the above mentioned range. The reasonable amounts of these adsorption preventors and stabilizing agents are dissolved and prepared as aqueous solution with suitable concentration and pH which can be used. The ratios of permeability pressure of this solutions are in the range of 0.1 to 3.1, preferentially 1.0. Since the stable range of TCF-II against pH is pH6 to 9, the aqueous solution for the preparation of pharmaceutical product is suitable to adjust pH to 6.0 to 50 9.0.

Then, the adsorption-prevention and stability of TCF-II in the pharmaceutical product in the present invention is further described in detail as shown in Experiments in the following:

Experiment 1. Test for prevention of adsorption

55

Human embryonic lung fibroblast, IMR-90 cells (ATCC, CCL186) were cultured for 7 days in DMEM containing 5% fetal bovine serum. 200 µg of TCF-II which was purified from the culture broth was dissolved in 100 ml of 0.01M phosphate buffer, pH7.0, containing 0.15 M NaCl (PBS). Each 0.5ml of the TCF-II

solution was added to glass tubes and polypropylene tubes. Separately, the solutions containing two-fold, concentration of each added material shown in Table 1a, b and c were prepared with PBS. 0.5ml of the above solution with each concentration of added material was added to each tubes containing 0.5 ml of the TCF-II solution, and mixed well. The final concentration of TCF-II is 1  $\mu\text{g/ml}$  and the final concentration of each added material is adjusted to concentration described in Table 1 a, b and c.

As a control, 0.5ml of PBS was added to each glass tube or polypropylene tube containing 0.5 ml of the TCF-II solution.

Each test was carried out duplicately, and TCF-II activity was determined after the incubation for 1 hour at 37 °C. Experimental values were shown as mean of duplicate incubations.

The activity of TCF-II was determined by cytotoxic activity as following.

A clone L929-18 with high sensitivity to TCF-II, which was obtained by subcloning mouse L929 (ATCC, CCL1), was used as a target cell.

L929-18 cells were grown to confluence in the Dulbecco's modified eagle medium (DMEM ) containing 10% FCS, then harvested by trypsin treatment and by centrifugation. The obtained cells were suspended at a cell density of  $6 \times 10^5$  cells/ml in DMEM containing 10% FCS and 1  $\mu\text{g/ml}$  of actinomycin D.

A serially diluted test sample was prepared by repeating dilution of test sample with DMEM which was used in the preparation of the cell suspension. Each 50  $\mu\text{l}$  of a serially diluted test sample was added to each well in 96 well-microplate ( Falcon).

50  $\mu\text{l}$  of the cell suspension was inoculated into each well containing a serially diluted test sample and the culture was carried out at 37 °C for 2 days in a CO<sub>2</sub> incubator. After the culture, the medium was removed gently and the cells were washed twice with PBS. The viable cells which adhere to each well were fixed and stained by addition of 50  $\mu\text{l}$  of 0.5% crystal violet in the mixture of methanol and water (1:4) to each well. Each well was washed with distilled water and dried, and crystal violet in each well was extracted with Sorenson's buffer (mixture of 6.1 ml of 0.1M dissodium citrate, 3.9ml of 0.1N HCl, and 10ml of ethanol). Absorbances of the extracts at 570 nm were determined by a microtiter spectrophotometer.

Unit of TCF-II ( $\mu\text{g/ml}$ ) was defined as the dilution ratio given 50% cell death and the residual cytotoxic activity after the incubation was estimated as the relative activity(%) against the activity (100%) determined immediately after the preparation of test sample.

The results in Table 2,a, b, and c showed that TCF-II was easily adsorbed on surface of glass and polypropylene vessels and the adsorption preventors using in the present invention effectively prevented TCF-II from the adsorption.

Table 2

(1) Glass tube

a. Effect of high molecular weight additives on  
adsorption of TCF-II.

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concentration	added material				
	human serum albumin(HSA)	low mol.w. gelatin*	gelatin	polyethylene glycol4000	dextran40
	.....residual relative activity (%).....				
0	8.3	8.3	8.3	8.3	8.3
0.01	16.7	8.3	25.0	8.3	-
0.05	25.0	8.3	37.5	8.3	8.3
0.1	50.0	16.7	50.0	16.7	-
0.25	100.0	16.7	100.0	16.7	8.3
0.50	100.0	33.3	100.0	16.7	8.3
1.00	100.0	50.0	100.0	16.7	12.5
2.00	100.0	100.0	100.0	33.3	12.5
10.00	100.0	100.0	100.0	-	75.0
20.00	100.0	100.0	100.0	-	-

\*:mean molecular weight = 6,000 (NIPPI Co. Ltd)

b. Effect of non-ionic detergents on adsorption of  
TCF- II

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added material

concentration (%)	added material	
	Tween80	Tween20
	.....residual relative activity (%).....	
0	8.3	8.3
0.0001	16.7	16.7
0.0005	25.0	25.0
0.001	50.0	50.0
0.005	100.0	100.0
0.01	100.0	100.0
0.05	100.0	100.0
0.10	100.0	100.0
1.00	100.0	100.0

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## (2) Polypropylene tube

c. Effect of human serum albumin and Tween 80 on  
adsorption of TCF-II

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	added material		added material	
	concentration (%)	human serum albumin (HSA)	concentration (%)	Tween 80
20	residual relative activity		residual relative activity	
	0	25.0	0	25.0
	0.01	50.0	0.0001	33.3
	0.10	75.0	0.0005	50.0
25	0.25	100.0	0.001	75.0
	0.50	100.0	0.005	100.0
	1.00	100.0	0.01	100.0
	2.00	100.0	0.05	100.0
30	10.00	100.0	0.1	100.0
	20.00	100.0	1.0	100.0

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## Experiment 2 Stability test

Under the conditions which prevented TCF-II from adsorption on surface of glass tube, effect of various kinds of added material on the stability of TCF-II was studied. 120  $\mu$ g of purified TCF-II from culture broth of IMR-90 cells was dissolved in 30 ml of PBS containing 0.02% Tween 80. After sterilization by filtrating the TCF-II solution using 0.22  $\mu$  filter, 0.5ml of the sterilized TCF-II solution was added to each sterilized glass tube.

The solution with two-fold concentration of each added material shown in Table 2 a, b and c, was prepared, and then sterilized by filtration with 0.22  $\mu$  filter. 0.5ml of the each solution was added to each glass tube containing 0.5ml of TCF-II solution and after mixing well, the glass tubes were sealed to prevent bacteria from contamination. As a control, 0.5 ml of PBS not containing Tween 80 was added to each glass tube containing 0.5 ml of TCF-II solution. The final concentration of TCF-II, Tween 80 and each added material were 2  $\mu$ g/ml, 0.01% and concentration shown in Table 3 a, b and c, respectively.

Each test was carried out duplicately. The TCF-II activity was determined after the incubation for one week at 40°C and estimated as relative activity (%) against the activity (100%) determined before the incubation. Experimental values were shown as mean of duplicate incubations.

From the results of Table 3 a, b and c, it was found that stabilizing agents which were used in the present invention had the effect which keeps on maintaining the activity of active component, TCF-II in the liquid state.

Table 3

a. Effect of high molecular weight additives on  
stability of TCF-II\*

10			storage period (day) at 40°C		
		added			
		concentration	0	3	7
		(%W/V)	.....residual relative activity (%).....		
15	human serum	0.0	100.0	25.0	16.7
	albumin	0.1	100.0	50.0	33.3
		0.25	100.0	100.0	100.0
20		0.5	100.0	100.0	100.0
	gelatin	0.0	100.0	25.0	16.7
		0.1	100.0	25.0	25.0
		0.25	100.0	100.0	100.0
25		0.5	100.0	100.0	100.0
	low mol.	0.0	100.0	25.0	16.7
	weight	0.5	100.0	25.0	16.7
30	gelatin	2.5	100.0	33.3	25.0
	(mean M.W.				
	6,000)				
		0.0	100.0	25.0	16.7
35	polyethylenglycol 4000				
		0.5	100.0	16.7	12.5
		2.5	100.0	16.7	12.5

\*:All test samples contained 0.01% Tween 80 to prevent TCF-II from adsorption  
on surface of glass tube.

## b. Effect of added carbohydrates on stability of TCF-E

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		storage period (day) at 40°C			
10		added			
		concentration	0	3	7
		(%W/V)	.....residual relative activity (%).....		
15	Dextran40	0	100.0	25.0	16.7
		2	100.0	25.0	8.3
		10	100.0	12.5	4.2
20	Sorbitol	0	100.0	25.0	16.7
		2	100.0	33.3	25.0
		10	100.0	66.7	66.7
		20	100.0	100.0	100.0
		40	100.0	100.0	100.0
25	Mannitol	0	100.0	25.0	16.7
		2	100.0	33.3	16.7
		10	100.0	66.7	50.0
		20	100.0	100.0	95.0
		40	100.0	100.0	100.0
30	Glucose	2	100.0	16.7	8.3
		10	100.0	12.5	4.2
		20	100.0	25.0	16.7
35	Fructose	2	100.0	12.5	6.3
		10	100.0	<2.0	<2.0
40	Mannose	0	100.0	25.0	16.7
		2	100.0	16.7	4.2
		10	100.0	<2.0	<2.0
45	Xylitol	0	100.0	25.0	16.7
		2	100.0	33.3	16.7
		10	100.0	100.0	66.7
		20	100.0	100.0	96.5

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## c. Effect of added amino acids on stability of TCF-II\*

	amino acid	added concentration (%)	storage period (day) at 40°C		
			0	3	7
			.....residual	relative activity (%)	.....
10	Arginine	0	100.0	25.0	16.7
		1	100.0	25.0	16.7
		5	100.0	50.0	33.3
15	Glycine	0	100.0	25.0	16.7
		1	100.0	33.3	25.0
		5	100.0	100.0	66.7
20	Lysine	10	100.0	100.0	100.0
		0	100.0	25.0	16.7
		1	100.0	25.0	16.7
25	Alanine	5	100.0	66.7	16.7
		0	100.0	25.0	16.7
		1	100.0	25.0	16.7
30		5	100.0	100.0	50.0
		10	100.0	100.0	90.0

\*:All test samples contained 0.01% Tween 80 to prevent TCF-II from adsorption on surface of glass tube.

Nextly, the results of pharmacological activities of TCF-II were shown in the following.

in vivo Antitumor Test to Sarcoma 180 of Human New Cytokine, TCF-II

Materials and methods

① Experimental animals

Female ICR mice of 7 weeks old were purchased from Charles River Japan Inc.

② Tumor cell lines

Sarcoma 180 cells were supplied by National Cancer Center, subcultured in mice with one time per week on this laboratory.

③ Test samples

Test samples were prepared by dissolving TCF-II in 0.01M phosphate buffer, pH7.0 containing 0.8% NaCl, 0.01% Tween 80 and 0.25% human serum albumin.

Two series of test samples composed of 0.2 µg TCF-II/0.2ml and 1.0 µg TCF-II/0.2ml were prepared. To analyze the effect of pyrogen, test sample (940 pg pyrogen/0.2ml) containing standard pyrogen (Difco Inc.) corresponding to the amounts of pyrogen in 1 µg of TCF-II was also prepared.

④ Acute toxicity test

Acute toxicity test was carried out, using two mice group. Two doses, 10 µg TCF-II/mouse and 20 µg TCF-II/mouse were administered by injecting them into tail vein of mice, respectively. Toxicity was determined from mortality of animals.

⑤ Antitumor test



Antitumor test was carried out , using seven mice/group.

Sarcoma 180 ( $10^6$  cells/mouse) were planted under the skin of ICR mouse. The mice were divided in each test group and the test samples were injected into tail vein for 7 days at one time/every day. Inhibitory effect of tumor growth was determined by inhibitory ratio

$$\frac{(C-T)}{C} \times 100\%$$

which was obtained from mean tumor weight (MTW) of injected groups against control group.

#### Results of tests

##### ① Acute toxicity test

Toxicity was not appeared at doses of both 10  $\mu$ g and 20  $\mu$ g TCF-II/mouse.

##### ② Antitumor test

Results after 3 weeks from the initiation of injection were shown in Table 4.

Table 4

Sample	Dose	MTW (mg)	$\frac{C-T}{C} \times 100\%$
control	0.0	3024.71	-
pyrogen	940 pg/mouse	3036.00	-0.37
TCF-II	0.2 $\mu$ g/mouse	1787.71	49.92
TCF-II	1.0 $\mu$ g/mouse	1984.21	34.40

Since on the above test, the optimum dose of TCF-II was unknown, test was not carried out by fitted doses. However, from these results, it was found that lower dose rather than high dose was much effective.

Moreover, anti-tumor effect by direct injection to intratumor were also studied by following method.

#### Methods:

Sarcoma 180 ( $1 \times 10^6$  cells/mouse) were planted under the skin of ICR mouse. The mice in which solid-tumor were vitally settled, were selected after one week of plantation. 0.2  $\mu$ g of TCF-II was injected for 7 days at one time/every day. When observation was continued for two weeks after the termination of injection, remarkable anti-tumor effect which caused necrosis of tumor site, changing into black was obtained. Moreover, mice in which tumor was disappeared, were observed.

#### Brief Description of the Drawings

Fig.1 shows an elution profiles of TCF-II and plasminogen activator from the culture broth of IMR-90 cells containing 5% calf serum on CM-Sephadex C-50 chromatography. (1) and (2) shows the fractions eluted with 0.05M Tris-HCl buffer, pH7.0, containing 0.3M NaCl and with 0.05M Tris-HCl buffer, pH7.0, containing 0.6M NaCl, respectively.

-○-, -●- and -◐- represent absorbance at 280 nm, plasminogen activator activity and cytotoxic activity to L929-18 cells, respectively.

Fig.2 shows the results of Con A affinity chromatography of TCF-II fraction which was eluted with 0.05M Tris-HCl buffer, pH 7.0, containing 0.6M NaCl on CM-Sephadex C-50 Chromatography of the culture broth of IMR-90 cells. (1) and (2) represent the washed fractions by 0.05M Tris-HCl buffer, pH7.0, containing 0.5M NaCl and the eluted fractions by 0.05M Tris HCl buffer ,pH7.0, containing 0.5M NaCl and 0.3M  $\alpha$ -methyl-D-mannopyranoside, respectively.

-●- and -○- represent absorbance at 280 nm and cytotoxic activity, respectively.

Fig. 3 shows an elution pattern on Mono S-HPLC of the TCF-II fraction which was obtained on Con A Sepharose affinity chromatography. -○- represents cytotoxic activity.

Fig.4 shows an elution profile of TCF-II on Heparin-sepharose affinity chromatography of the elute from Mono S-HPLC. (1) and (2) represent the washed fraction by 10mM Tris-HCl buffer, pH7.5, containing 0.3MNaCl and the eluted fraction by NaCl gradient from 0.3 to 2.0M, respectively. -●- and -○- represent absorbance at 280nm and cytotoxic activity, respectively.

Fig.5 shows SDS electrophoresis of TCF-II (reduction and non-reduction).

Fig.6 shows heat stability of TCF-II.

Fig.7 shows pH stability of TCF-II.

Fig.8 shows cytotoxic activity of TCF-II to human tumor cell lines in vitro.

Fig.9 shows cytotoxic activity of TCF-II to Sarcoma 180.

Fig.10 shows cytotoxic activity of TCF-II to Meth A and P388, respectively. -●- and -○- represent M eth A and P388, respectively.

Fig.11 shows cytostatic activity of TCF-II to human tumor cell lines. -●- and -○- represent cytostatic activities to ovarian carcinoma, BG-1 and breast carcinoma, MCF-7, respectively.

Fig.12 and Fig.13 show effect of TCF-II on incorporation of  $^3\text{H}$ -thymidine into the lymphocytes in the mixed lymphocytes culture at day 5 and at day 8, respectively. Six samples at day 5 and at day 8 were determined, respectively. Results are given as mean  $\pm$ SD.

Fig.14 shows the stimulatory effect of TCF-II on the growth of vascular endothelial cells, HUVEC.

Fig.15(1) and (2) show the base sequence of TCF-II cDNA and the amino acid sequence of TCF-II, which was deduced from the base sequence, respectively.

Fig.16 shows the comparison between the amino acid sequence of TCF-II, which was deduced from the above base sequence and an amino acid sequence of hHGF reported by Miyazawa et al.

## A BEST FORM TO PUT ON THE INVENTION

The present invention is further described concretely as shown in EXAMPLES in the following:

### EXAMPLE 1

#### (1) Culture of Human Fibroblast, IMR-90 cells

Human fibroblast, IMR-90 cells(ATCC CCL 186) ( $3 \times 10^5$  cells) were inoculated in one liter-roller bottle holding 100ml DMEM containing 5% calf serum (CS), and were cultured for 7 days with rolling at the rates of 0.5 to 2 rpm. When the total cell numbers reached  $1 \times 10^7$  cells, the cells were harvested by trypsin treatment and collected in bottom of the bottle. After adding 250 ml of fresh DMEM containing 5% CS to the bottle, 100g of autoclaved ceramics with 5 to 9 mesh (Toshiba Ceramic. Co. Ltd) was added to the bottle containing cell suspension. Standing culture was carried out at  $37^\circ\text{C}$  for 24 hours. Then 250 ml of DMEM containing 5% CS was added to the bottle (final medium volume was 500 ml), and the culture was continued. Every 7 to 10 days all amount of the medium (about 500 ml) was collected and fresh medium (500ml) was supplied. Thus, the production was continued for 2 months and 4 l of the culture broth were collected per roller bottle. Specific activity of thus obtained culture broth was 32 u/ml.

#### (2)Purification of the Glycoprotein, TCF-II

75 liters of the culture broth described in (1) was concentrated about 10-fold by UF concentration, using Amikon-membrane filter (pore size, M.W. 6,000). Then, after CM-Sephadex C-50 was equilibrated with 0.05M Tris-HCl buffer, pH7.0, 1.5kg of wet weight of the resins was added to the above UF concentrated broth and the substance was absorbed to the resins by gently stirring at  $4^\circ\text{C}$  for 24 hour under pH 6.5 to 7.0. After the absorption, the resins were collected by filtrating through a Whatman No.2 filter paper. The collected resins were washed with 0.05M Tris-HCl buffer, pH7.0. Approximately 1500g of the washed resins was packed to the column ( $\varnothing$  7 $\times$ 40cm), and the column was eluted with 0.05M Tris-HCl, pH7.0, containing

0.01% Tween 20 and 0.3M NaCl. Elution of protein was monitored by absorbance at 280 nm. When protein was eluted almost completely, further elution at salt concentration of 0.6M NaCl was carried out. Cytotoxic activity to L929-18 cells and tissue plasminogen activator (t-PA) activity which IMR-90 cells produce were determined in each fraction. Thus obtained elution pattern is shown in Fig.1. The fraction which was eluted at salt concentration of 0.6M NaCl exhibited the potent cytotoxic activity. This fraction was defined as TCF-II fraction. Then, Con A-Sepharose CL-6B (Pharmacia) was equilibrated with 0.05M Tris-HCl buffer, pH7.0, containing 0.5M NaCl, and the gel was packed into the column ( $\varnothing$  2.5x8cm). The column was washed well with the same buffer, the TCF-II fraction which was eluted from the CM-Sephadex column was loaded on the column. After the column was washed again with 0.05M Tris-HCl, pH7.0, containing 0.5M NaCl with 10 times volume of the column bed volume, the substance was eluted with 0.05M Tris-HCl, pH7.0, containing 0.5M NaCl and 0.3M  $\alpha$ -methyl-D-mannopyranoside at a flow rate of 70 ml/hour. Elution of protein was monitored by optical absorbance at 280 nm and cytotoxic activity in each fraction was determined. Thus obtained elution pattern is shown in Fig.2. The fraction which was eluted firstly was collected and dialyzed against distilled water for 48 hours. The dialyzed fraction was lyophilized and white powder was obtained. The lyophilized powder was dissolved with a small volume of 0.05M Tris-HCl buffer, pH7.0, containing 0.01% Tween 20 and 0.2M NaCl, and was loaded on MonoS column (Pharmacia) for HPLC which was equilibrated with 0.01M phosphate buffer, pH 7.0, containing 0.01% Tween 20. After the column was washed with 0.01M phosphate buffer, pH7.0, containing 0.01% Tween 20 for 20 min at a flow rate of 0.5ml/min, elution was carried out by the NaCl gradient from 0 to 1.0M in 60min. The obtained elution pattern is shown in Fig.3. The active fraction was eluted at 0.76M NaCl. The active fraction was collected and diluted with the equilibration buffer and then loaded on the Mono S column for HPLC again. Elution was carried out again by the NaCl gradient from 0 to 1.0 M NaCl in the same buffer. Heparin-Sepharose (Pharmacia) was equilibrated with 10 mM Tris buffer, pH 7.0, containing 0.3M NaCl, and 5ml of the gel was packed to the column ( $\varnothing$  1.0x7cm). The active fraction from Mono S-HPLC was collected, diluted with 0.01M Tris-HCl buffer, pH7.0, to NaCl concentration of 0.3M, and loaded on the above mentioned column. Then, the column was washed with 0.01M Tris-HCl pH7.0, containing 0.3M NaCl with 10 times volumes of the column bed volume. The substance was eluted at a flow rate of 20 ml/hour by the NaCl gradient from 0.3M to 2.0 M in the same buffer. The elution pattern is shown in Fig.4. Thus, the purified glycoprotein was obtained. As shown in Table 5, 0.12 mg of the active glycoprotein was obtained from 75 liters of the culture broth as the starting material. This glycoprotein was a tumor cytotoxic factor and its specific activity was 5,248,000 u/mg.

Table 5

Purification of tumor cytotoxic factor obtained from the culture broth  
of IMR-90 (containing 5% CS)  
TCF-II: A fraction eluted with 0.6M NaCl on Sephadex C-50 chromatography

Purification Step	Volume (ml)	Protein (mg/ml)	Total Protein (mg)	Cytotoxic Activity (u/ml)
Culture broth	75000	3.30	247500.0	32.0
UF Concentration	10000	23.40	234000.0	192.0
CM-Sephadex chromatography (eluted fraction with 0.6M NaCl)	894	0.32	205.6	2048.0
ConA Sepharose chromatography	244	0.26	63.4	5120.0
Mono S-HPLC	13	0.26	2.1	80000.0
Heparin-Sepharose chromatography	6	0.02	0.12	104960.0

Total Activity ( $\times 10^4$ u)	Specific Activity (u.mg)	Purification Factor	Recovery (%)
24.0	9.7	1.0	100.0
192.0	8.2	0.9	80.0
183.1	8904.3	918.0	76.3
124.9	19692.3	2030.0	52.0
104.0	500000.0	51546.4	43.3
56.0	5248000.0	541030.9	23.3

Physicochemical properties of TCF-II obtained by the above mentioned procedure are shown as follows.

① Determination of molecular weight on SDS gel electrophoresis

Molecular weight of TCF-II was determined by electrophoresis using polyacrylamide gel containing 0.1% SDS. The glycoprotein showed two adjacent bands with M.W. 78,000 and 74,000. When the glycoprotein was reduced by 2-mercaptoethanol and the similar electrophoresis was carried out, three bands with molecular weight of 52,000, 32,000, and 28,000 were observed (Fig.5).

These results indicate that TCF-II is a heterodimer composed of a common subunit with M.W. 52,000 and a subunit of M.W. 28,000 or a subunit of M.W. 32,000.

② Isoelectric point

Isoelectric point of the substance was determined as 7.4 to 8.55 by isoelectric focussing, using Phast Gel IEF3-9.

### ③ Heat stability

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TCF-II which had the activity of 51,200 u/ml was added to 0.1M Tris-HCl buffer, pH7.0, containing 0.01% Tween 20 and the TCF-II solution with 512 u/ml was prepared. The solution was treated for 10 min at 25, 35, 50, 60, 70 80, 90 , and 95 ° C. The residual cytotoxic activity (u/ml) after treatment at each temperature was estimated as the relative activity (%) against the activity (u/ml, 100%) at 25 ° C (control). As shown in Fig.6, the substance was stable till 60 ° C.

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### ④ pH stability

Each buffer containing 0.01% Tween 20, composition of which was shown in Table 6, was prepared. The amount of TCF-II corresponding to 51,200 u/ml when it was prepared at pH 8 was dissolved in each buffer, and allowed to stand at 37 ° C for 1 hour. The relative activity (%) compared to the control at room temperature for one hour was determined. As shown in Fig. 7, the glycoprotein was stable in the range of pH 6 to 9.

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Table 6 Preparation of buffers

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pH1-3	1/10 M	Glycine-HCl
pH4-6	1/10 M	Acetate buffer
pH7-8	1/10 M	Tris-HCl
pH9.12	1/10 M	Glycine-NaOH

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### ⑤ N-terminal amino acid sequence of TCF-II.

50 µg of TCF-II was reduced and three polypeptides, A with M.W. 52,000, B with M.W.32,000, and C with M.W. 28,000, were separated by the electroblot method. Amino acid sequence of each polypeptide was analyzed using the Applied Biosystems 477A Protein Sequencer. N-terminal amino acid sequence of polypeptide A could not be determined because its N-terminus had been blocked. Polypeptides B and C had the common N-terminal amino acid sequence as follows;

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Val-Val-Asn-Gly-Ile-Pro-Thr-X-Thr-Asn-Ile-Gly-

1 2 3 4 5 6 7 8 9 10 11 12

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X-Met-Val-Ser-Leu-

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13 14 15 16 17

X means an unidentified amino acid.

Since polypeptide B and C show the same N-terminal amino acid sequence, TCF-II appears to have dimer structure in which polypeptide A with M.W. 52,000 is bound to polypeptide B with M.W. 32,000 or to polypeptide C with M.W. 28,000 by S-S bridge.

5 ⑥ Amino acid composition

Ten  $\mu$ g of TCF-II, which was determined by BioRad Protein Assay kit, was hydrolyzed with HCl and then its amino acid composition was determined using a Hitachi Amino Acid analyzer, Model L-8500.

Amino acid composition of the material is shown as follows.

10 Amino acid composition:

	A.A	nmol	mol%
15	Asp	10.375	12.97
	Glu	7.750	9.69
	Ser	5.000	6.25
20	Gly	7.250	9.06
	His	3.000	3.75
	Arg	5.375	6.72
25	Thr	5.125	6.41
	Ala	2.625	3.28
30	Pro	5.625	7.03
	Tyr	3.875	4.84
	Val	4.125	5.16
35	Met	1.875	2.34
	Cys	ND	-
40	Ile	5.000	6.25
	Leu	4.875	6.09
	Phe	2.250	2.81
45	Trp	ND	-
	Lys	5.875	7.34
50	total	80.000	100(99.99)

55 EXPERIMENT 2

Cytotoxic activity to the tumor cells of the glycoprotein, TCF-II obtained in EXPERIMENT 1 is shown.

Cell growth inhibition (%) of TCF-II was calculated compared to the group in the absence of TCF-II as the control, and plotted against the concentration of TCF-II. As shown in Fig.8, TCF-II showed the potent inhibition of the growth of KB and HeLa cells whereas it did not inhibit the growth of normal cells, IMR-90.

TCF-II was dissolved in DMEM containing 10% FCS to the concentration of 320 u/ml. The titer of anti-LT antibody which neutralized 1000 u/ml LT wash added to the TCF-II solution and the mixture was allowed to stand at 37° C for 1 hour. Similarly, anti-TNF antibody and anti-INF- $\beta$  were added to the TCF-II solution at the concentrations of  $1 \times 10^6$  u/ml and 1000 u/ml, respectively. Each antibody used in this experiment is commercially available.

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EXPERIMENT 3

30 Sarcoma 180. Meth A sarcoma and P-388 cells were used as mouse tumor cell lines.

Sarcoma 180 cells were suspended in DMEM containing 10% fetal bovine serum and Meth A and P-388 cells were suspended at  $2 \times 10^4$  cells/ml, in RPMI 1640 medium containing 10% fetal bovine serum, respectively. 50  $\mu$ l of each cell suspension was inoculated into each well in 96-well microplate (Falcon). TCF-II was dissolved in DMEM containing 10% fetal bovine serum for Sarcoma 180 cells and in RPMI 1640 medium containing 10% fetal bovine serum for Meth A and P-388 cells. Each 50  $\mu$ l of TCF-II solution which was prepared so that the final concentrations of TCF-II were 0, 2, 4, 8, 16, 31, 62, 125, 250, 500 and 1000 ng/ml, respectively, was added to each well containing each cell suspension. After each cell line were cultured at 37 °C for 3 days in a CO<sub>2</sub> incubator. The cells in each well were stained with Trypane blue and the viable cell number were counted using a heamacytometer. The viable cell numbers were expressed as mean of duplicate experiments. Cytotoxic activity (%) was calculated according to the following equation.

$$\text{Cytotoxic activity(\%)} = \frac{\text{Average surviving numbers in control (cells/ml)} - \text{Average surviving cell numbers in TCF-II group (cells/ml)}}{\text{Average surviving cell numbers in control (cells/ml)}} \times 100$$

23

All cell lines were highly sensitive to TCF-II, and the IC<sub>50</sub> values for cytotoxic activity of TCF-II on Sarcoma 180, Meth A and P-388 cells were 6, 40 and 460 ng/ml, respectively.

#### EXPERIMENT 4

Inhibitory effect of the glycoprotein, TCF-II obtained in Experiment 1 on human tumor cell lines, ovarian carcinoma, BG-1 and breast carcinoma, MCF-7 is shown in the following.

BG-1 and MCF-7 cells were suspended in McCoy medium containing 10% FCS and in Eagle's MEM containing 10% FCS, non-essential amino acid mixture, pyruvate and Eagle's salts at  $2 \times 10^4$  cells/ml, respectively. TCF-II was dissolved in McCoy medium containing 10% FCS for BG-1 cells and in Eagle's MEM containing 10% FCS for MCF-7 and a serially diluted TCF-II solution was prepared by repeating 2-fold dilution of 4  $\mu$ g TCF-II/ml with the same medium.

50  $\mu$ l of each cell suspension was inoculated into each well in 96-well microplate (Falcon) Each 50  $\mu$ l of a serially diluted solution of TCF-II which was prepared for each cell line was added to the wells containing each cell suspension. The culture was carried out at 37°C for 5 days in CO<sub>2</sub> incubator. After the culture, the culture broth was removed and the cells were gently washed twice with PBS. The surviving cells which adhered to each well were fixed and stained by addition of 50  $\mu$ l of 0.5% crystal violet solution in the mixture of methanol and water (1:4) to each well. Each well was washed with distilled water and dried, and crystal violet in each well was extracted with Serenson's buffer. Absorbances of the extracts at 570 nm were determined by a microtiter spectrophotometer. Cell growth inhibition (%) was calculated for each cell line according to the following equation compared to the control in the absence of TCF-II.

Cell growth inhibition(%) =

$$\frac{\text{Control OD}_{570\text{nm}} - \text{TCF-II group OD}_{570\text{nm}}}{\text{Control OD}_{570\text{nm}}} \times 100$$

Results are shown in Fig.11. These results indicate that TCF-II inhibits the growth of both tumor cell lines, BG-1 and MCF-7.

#### EXPERIMENT 5

Promyelocytic leukemia cell line, HL-60 cells-differentiation inducing activity of the glycoprotein, TCF-II obtained in EXPERIMENT 1 is shown.

HL-60 cells were suspended at a cell density of  $3.5 \times 10^5$  cells/ml in RPMI 1640 medium containing 10% fetal bovine serum . 100  $\mu$ l of the cell suspension was added to each well in 96-well flat bottomed microplate(Falcon). Then, 100  $\mu$ l of the TCF-II solution in the same medium was added to each well containing cell suspension to give the final concentrations of 15.6, 62.5,125, 250, 500, and 1000 ng/ml.

The cells were cultured at 37°C for 3 and 7 days, and HL-6 cells-differentiation inducing activity of TCF-II was determined by nitroblue tetrazolium (NBT) reduction assay. Moreover, morphological change of the cells was observed.

##### 1)NBT reducing ability

NBT reducing ability is shown in Table 7.



Table 7

TCF-II	NBT reducing ability(%)	
	Culture days	
concentration (ng/ml)	3	7
0	7.4	11.7
15.6	10.6	20.4
62.5	11.1	24.5
125	12.4	28.3
250	16.9	45.2
500	12.4	29.6
1000	12.1	26.8

The values shown in Table 7 represent percentage of cells containing blue-black formazan deposits (average of 2 experiments) when at least more than 200 cells were counted.

(When HL-60 cells differentiate to normal cells, they get NBT reducing ability and accumulate blue-black formazan in them)

This result has demonstrated that TCF-II induces the differentiation of promyelocytic leukemia cell line, HL-60 and has the highest activity for the differentiation induction at 250ng/ml.

## 2) Morphological change

It is known that HL-60 differentiates to two ways of macrophages and granulocytes by differentiation inducing factors.

Changes in morphology and nucleus of the cells which were cultured with TCF-II at 37°C for 7 days were investigated by the light Giemsa staining method. The result indicates that HL-60 differentiates to granulocyte-like cells.

## EXPERIMENT 6

Stimulatory effect on cellular immunology of the glycoprotein, TCF-II obtained in EXPERIMENT 1 is shown.

The effect of TCF-II on blastogenic transformation of lymphocytes was observed when mixed lymphocyte culture was carried out in the presence of TCF-II.

Lymphocytes were isolated from human peripheral blood by the method of Ficoll-Conray and suspended in RPMI 1640 containing 10% FCS. Lymphocytes from two persons were mixed as 1:1 and added to wells in 96 well round bottomed microplate at  $1 \times 10^5$  cells/ 100  $\mu$ l/well. TCF-II was added at various concentrations and the cells were cultured in RPMI 1640 containing 10% FCS in a CO<sub>2</sub> incubator. <sup>3</sup>H-Thymidine was added at 0.25  $\mu$ Ci/well at 16 hours before the end of culture. After the culture the cells were harvested by a cell harvester and washed with PBS. The radioactivity of <sup>3</sup>H-Thymidine incorporated into the

cells were determined by a scintillation counter.

Results are given in Fig.12 and Fig.13. TCF-II did not show the stimulatory effect at day 5 of the culture as shown in Fig.12, but  $^3\text{H}$ -thymidine incorporation was significantly stimulated by the presence of TCF-II compared to the control at day 8 as shown in Fig.13. These results indicate that TCF-II stimulates the growth of cytotoxic T cell, that is, TCF-II possesses the enhancing effect on cellular immunology.

#### EXPERIMENT 7

Stimulatory effect on vascular endothelial cell growth of the glycoprotein, TCF-II obtained in EXPERIMENT 1 is shown.

Human umbilical vein endothelial cells, HUVEC was used as a test cell. The endothelial cells, HUVEC, were suspended at a cell density of  $2.5 \times 10^4$  cells/ml in E-GM medium containing 2% fetal bovine serum. 50  $\mu\text{l}$  of the cell suspension was added to each well in 96-well flat bottomed microplate (Falcon). Then, 50  $\mu\text{l}$  of the TCF-II solution which was prepared in the same medium was added to give the final concentrations of 0, 4, 8, 16, 31, 62, 125, 250, 500 and 1000 ng/ml. The cells were cultured at  $37^\circ\text{C}$  for 6 days in a  $\text{CO}_2$  incubator. After the culture, the culture medium in each well was removed and the cells were washed gently with PBS. The cells were removed from the well by trypsin treatment and viable cell numbers were counted using a hemacytometer.

The effect of TCF-II on human normal vascular endothelial cells is shown in Fig.14. The result indicates that TCF-II does not show the cytotoxic activity to the normal cells but has the stimulatory effect on the growth. Especially, the cell growth stimulatory activity was the maximal at a concentration of 125  $\mu\text{g/ml}$ .

The following Experiments show prescriptions of the pharmaceutical product in the present invention.

#### EXPERIMENT 8

TCF-II	20 $\mu\text{g}$
Human serum albumin	100mg

The above mentioned compositions were dissolved in 0.01M phosphate buffer, pH7.0, containing 0.15M NaCl (PBS) and the resulted solution was filled up to 20ml with PBS. After sterilization by filtrating the obtained solution using 0.22 $\mu$  filter, 2ml of the sterilized solution was poured into each vial tube and lyophilized, and then the vial tubes were sealed.

#### EXPERIMENT 9

TCF-II	40 $\mu\text{g}$
Tween80	1mg
Human serum albumin	50mg

The above mentioned compositions were dissolved in physiological salt solution (0.8%NaCl) and the solution was filled up to 20 ml with the same solvent. After the sterilization by filtrating the solution using 0.22 $\mu$  filter, 2ml of the sterilized solution was poured into each vial tube and lyophilized, and then the vial tubes were sealed.

#### EXPERIMENT 10

TCF-II	20 $\mu\text{g}$
Tween80	2mg
Sorbitol	4g

The above mentioned compositions were dissolved in PBS, and the resulted solution was filled up to 20 ml with PBS. After sterilization by filtrating the solution using 0.22 $\mu$  filter, 2ml of the sterilized solution was

poured into each vial tube and lyophilized, and then the vial tubes were sealed.

#### EXPERIMENT 11

5

TCF-II	40 $\mu$ g
Tween80	2mg
Glycine	2g

10

The above mentioned compositions were dissolved in physiological salt solution (0.8%NaCl), and the resulted solution was filled up to 20 ml with the same solvent. After sterilization by filtrating the solution using 0.22 $\mu$  filter, 2ml of the sterilized solution was poured into each vial tube and lyophilized, and then the vial tubes were sealed.

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#### EXPERIMENT 12

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TCF-II	40 $\mu$ g
Tween80	1mg
Sorbitol	2g
Glycine	1g

25

The above mentioned compositions were dissolved in physiological salt solution (0.8%NaCl), and the resulted solution was filled up to 20 ml with the same solvent. After sterilization by filtrating the solution using 0.22 $\mu$  filter, 2ml of the sterilized solution was poured into each vial tube and lyophilized, and then the vial tubes were sealed.

#### EXPERIMENT 13

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TCF-II	20 $\mu$ g
Sorbitol	4g
Human serum albumin	50mg

The above mentioned compositions were dissolved in PBS and the resulted solution was filled up to 20 ml with PBS. After sterilization by filtrating the solution using 0.22 $\mu$  filter, 2ml of the sterilized solution was poured into each vial tube and lyophilized, and then the vial tubes were sealed.

#### EXPERIMENT 14

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TCF-II	40 $\mu$ g
Glycine	2g
Human serum albumin	50mg

The above mentioned compositions were dissolved in physiological salt solution (0.8%NaCl), and the resulted solution was filled up to 20 ml with the same solvent. After sterilization by filtrating the solution using 0.22 $\mu$  filter, 2ml of the sterilized solution was poured into each vial tube and lyophilized, and then the vial tubes were sealed.

#### Industrial Availability

The present invention provides a new glycoprotein. The glycoprotein in the present invention can be used as a tumor cytotoxic factor, leukemia cell line-differentiation inducing factor, cellular immunology

enhancing factor and vascular endothelial cell growth factor and so on, and can be provided usually.

Moreover, the glycoprotein in the present invention can be used as a biochemical or pharmacological reagent.

## 5 Claims

1. A glycoprotein which is obtained from the culture broth of human derived fibroblasts and which is characterized by the following physicochemical properties,

a. Molecular weight; On the determinations of molecular weight by SDS gel electrophoresis, 78,000±2,000 or 74,000±2,000 under the nonreduced conditions and a common band A with 52,000±2,000 and band B with 30,000±2,000 or band C with 26,000 ± 2,000 under the reduced conditions.

b. Isoelectric point; 7.4 to 8.6.

c. Heat stability; Stable in heating at 60 ° C for 10min.

d. pH stability; Stable in the range of pH6 to 9.

e. Carbohydrate chain; Adsorbed to a Concanavalin A (Con A)-Sephadex column.

f. Biological activity; Inhibits the growth of KB cells, HeLa cells, and L-929 cells but not IMR-90 cells.

g. Reactivity to antibodies; The cytotoxic activity is not neutralized by anti-TNF antibody, anti-lymphotoxin antibody, and anti-interferon-β antibody.

h. N-terminal amino acid sequence; Above mentioned band B and band C are subchains of band A, respectively. N-terminus of band A is blocked. Band B and band C has common N-terminal amino acid sequence as follows;

Val-Val-Asn-Gly-Ile-Pro-Thr-

or

Val-Val-Asn-Gly-Ile-Pro-Thr-X-Thr-Asn-Ile-Gly-X-Met-

Val-Ser-Leu-

X means an unidentified amino acid.

2. A pharmaceutical product of biological factor obtained from human-derived fibroblast, which contains the glycoprotein according to claim 1 as active component, moreover one or more of proteins nonionic detergents as adsorption preventor, or one or more of proteins, carbohydrates and amino acids as stabilizing agent, and which is characterized by the following biological activity

① An antitumor activity:

Inhibits the growth of KB, HeLa, MCF-7 and BG-1 which are human-derived tumor cell lines, and have a cytotoxic activity to mouse L-929 cells, and to Sarcoma 180, Meth A sarcoma and P388 which are mouse tumor cell lines, but not inhibit the growth of IMR-90 cells which are human-normal cells.

② A leukemia cell differentiation inducing activity:

Induces the differentiation of the human-derived leukemia cell line, HL-60 to granulocyte-like cells.

③ A cellular immunology enhancing activity:

Stimulates the growth of human cytotoxic T cells

④ A vascular endothelial cell growth stimulating activity:

Stimulates the growth of human umbilical vein endothelial cells, HUVEC.

⑤ A hepatocyte growth stimulating activity:

Stimulates the growth of adult rat hepatocytes.

3. A pharmaceutical product of biological factor according to claim (2), in which the protein selected as the adsorption preventor is either albumin or gelatin.

4. A pharmaceutical product of biological factor according to claim (2), in which the nonionic detergent selected as the adsorption preventor is either Tween 80 or Tween 20.

5. A pharmaceutical product of biological factor according to claim (2), in which the protein selected as

the stabilizing agent of the pharmaceutical product is either albumin or gelatin.

6. A pharmaceutical product of biological factor according to claim (2), in which the carbohydrate selected as the stabilizing agent of the pharmaceutical product is sorbitol, mannitol or xylitol.
- 5 7. A pharmaceutical product of biological factor according to claim (2), in which the amino acid selected as the stabilizing agent of the pharmaceutical product is either glycine or alanine.
8. A pharmaceutical product of biological factor according to claim (2), which contains various combination  
10 of one or more of proteins or nonionic detergents as adsorption preventor, and one or more of proteins, carbohydrates or amino acids as stabilizing agent.
9. A DNA containing base sequence encoding TCF-II.
- 15 10. A DNA containing base sequence encoding amino acid sequence showed in Figure 15.
11. A cDNA containing base sequence encoding amino acid sequence showed in Figure 15.
12. A cDNA sequence containing base sequence showed in Figure 15.

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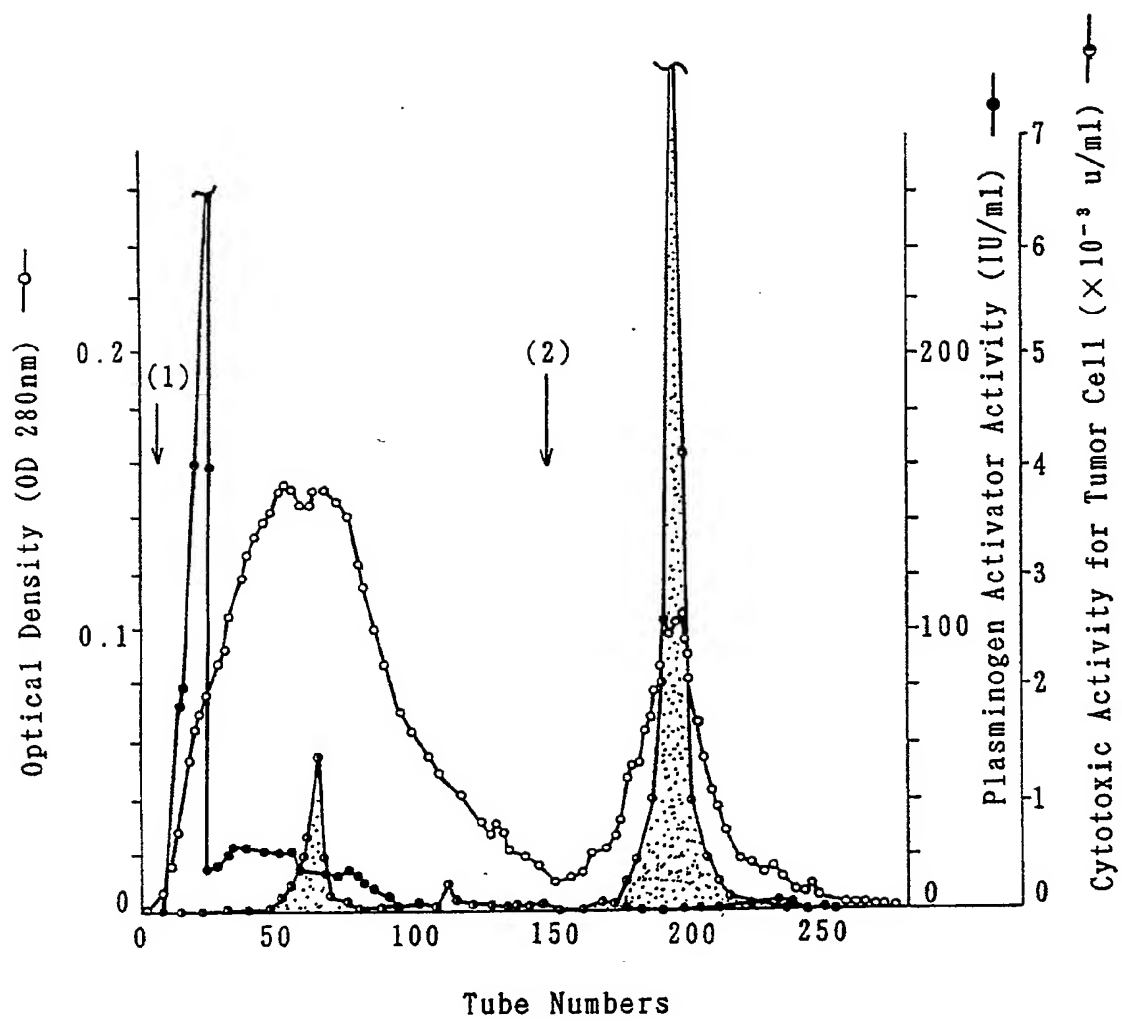
40

45

50

55

FIG. 1



F I G . 2

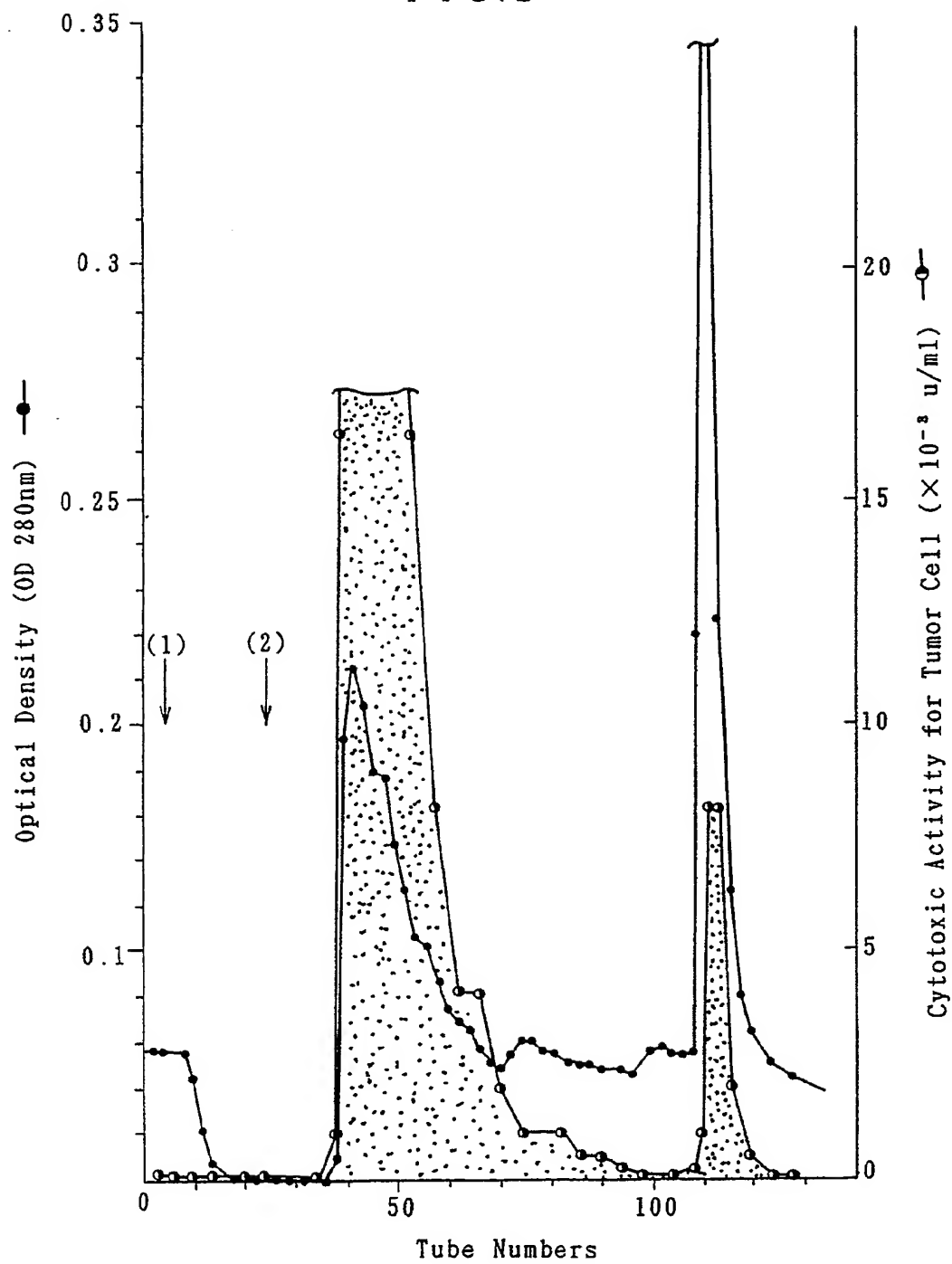


FIG. 3

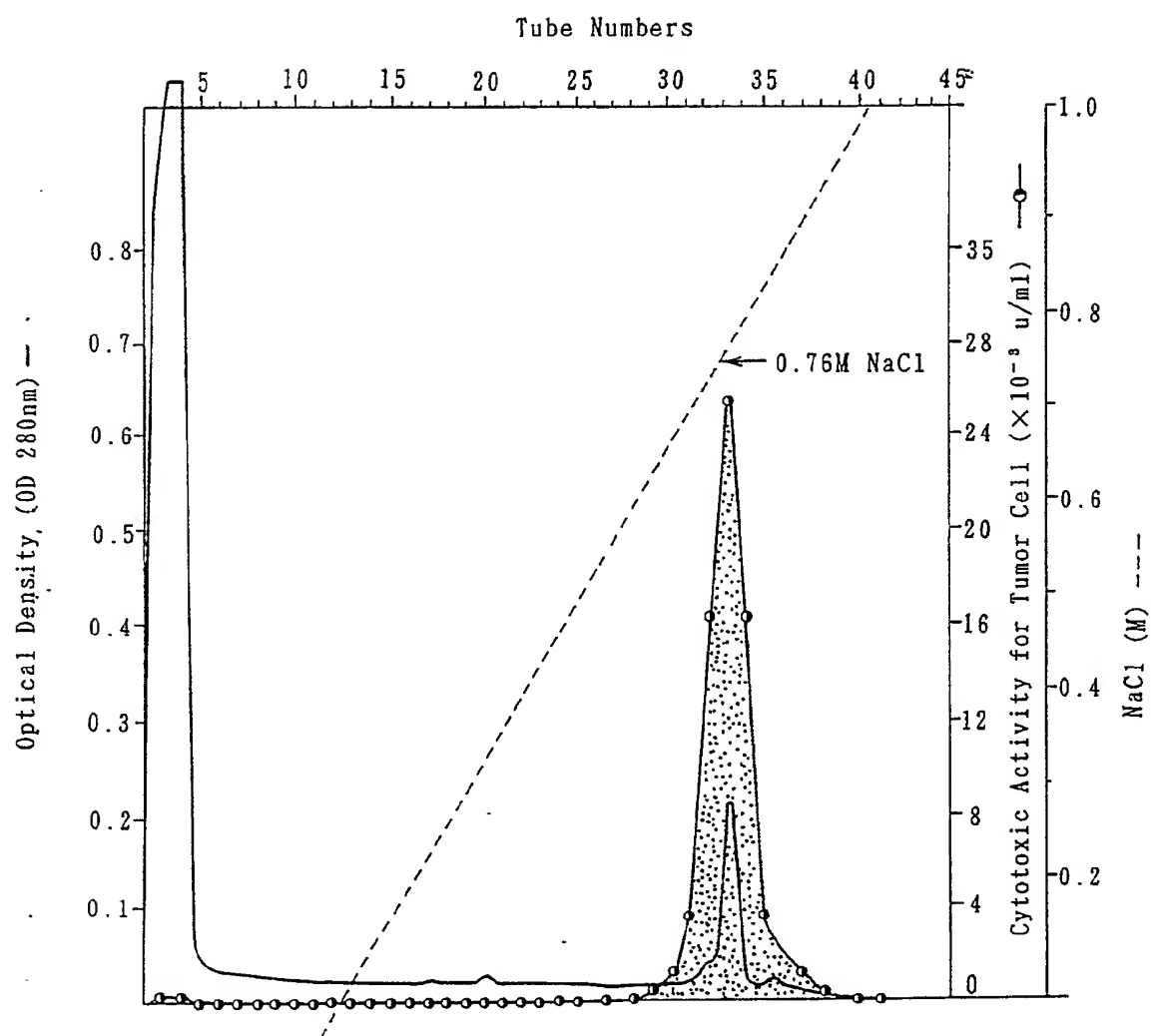




FIG. 4

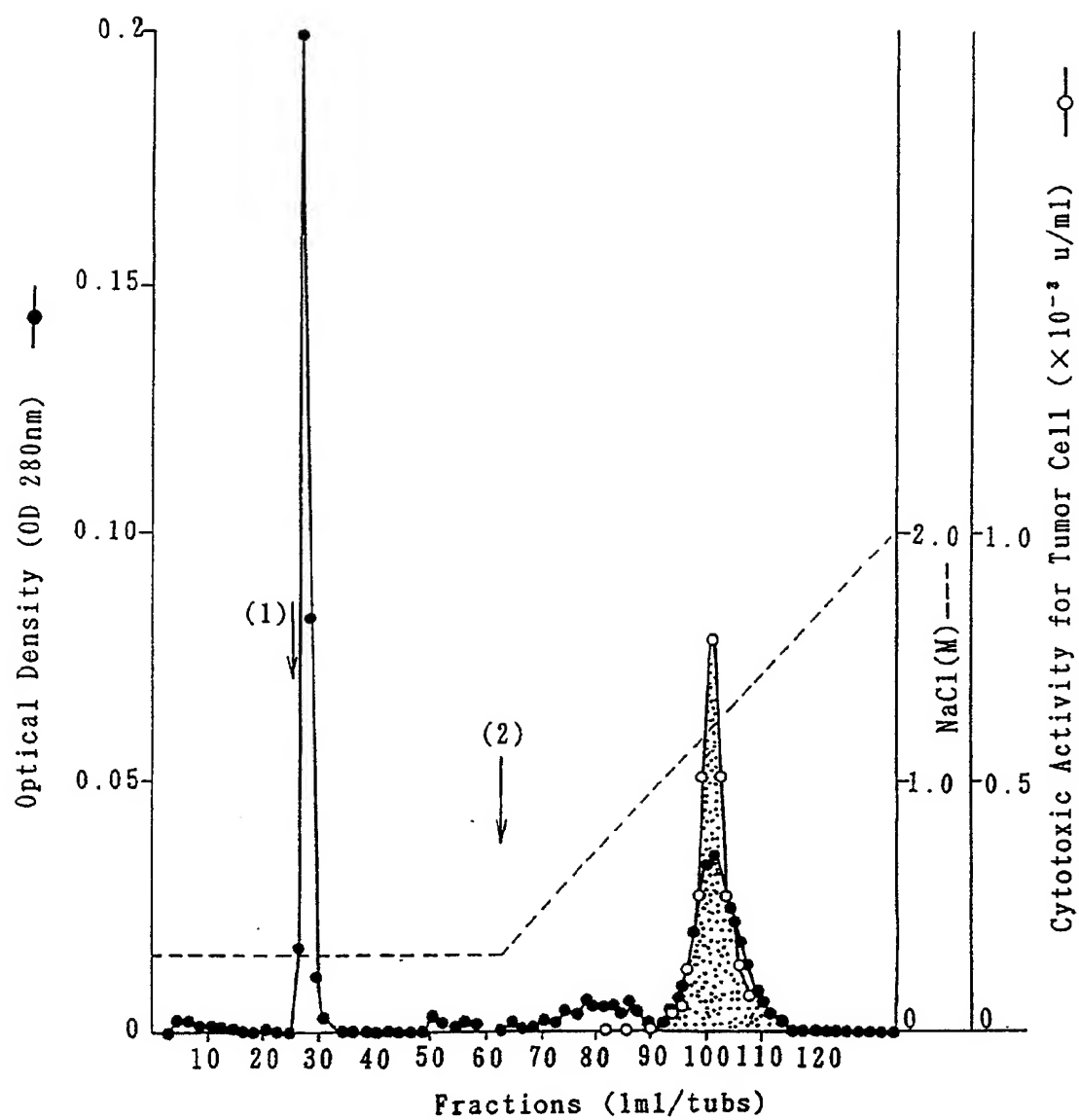
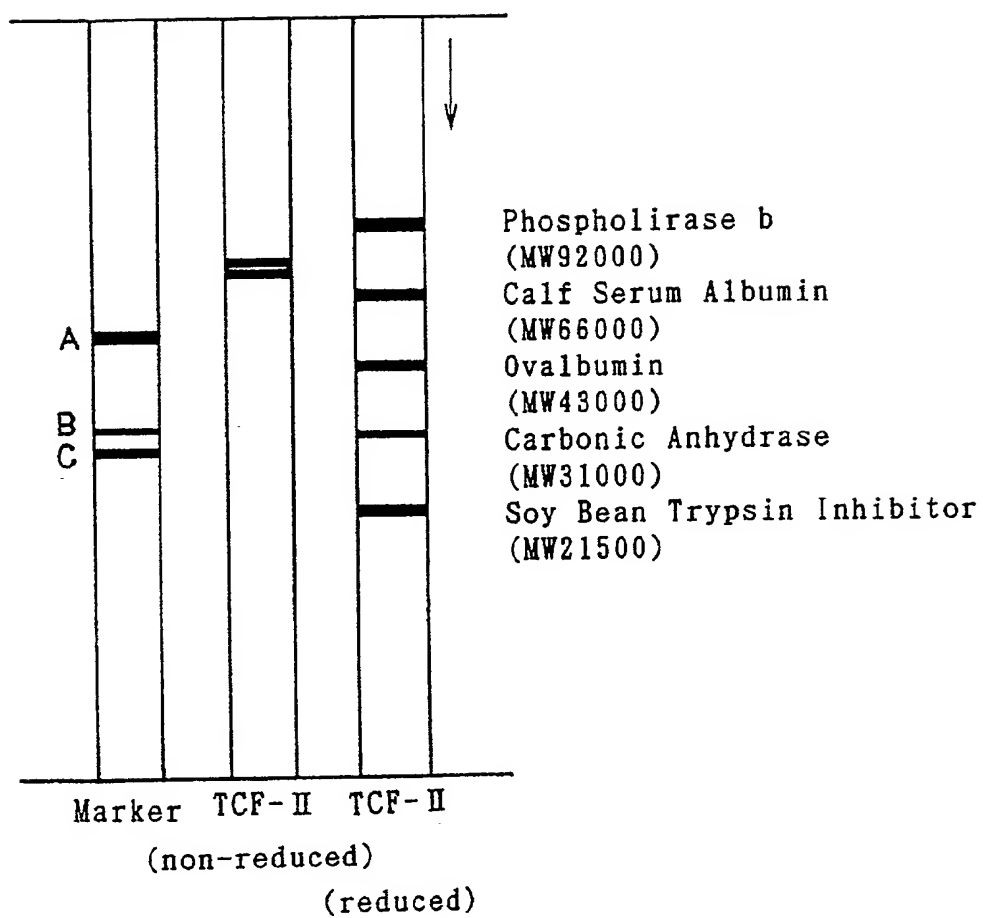
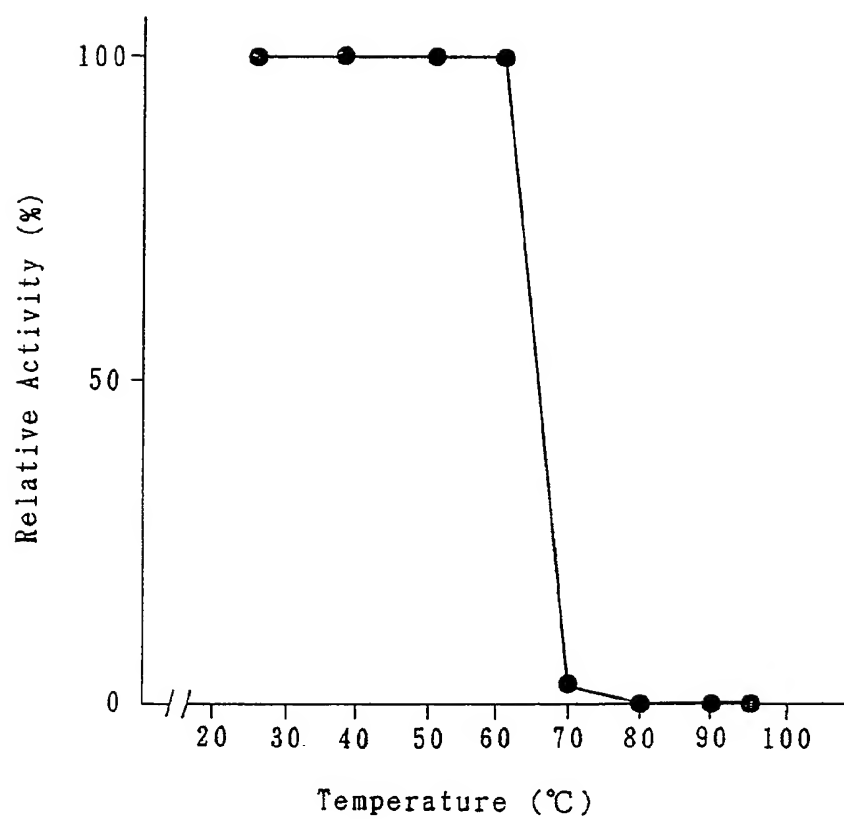


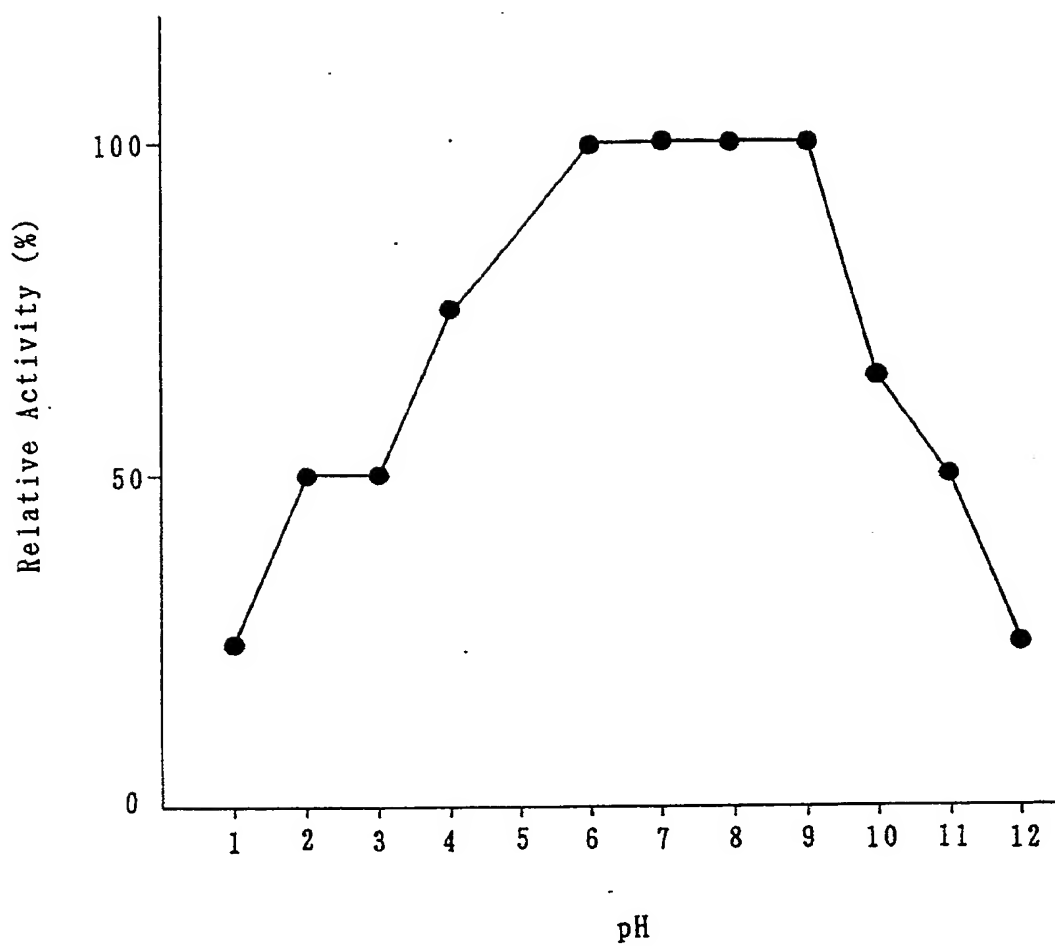
FIG. 5



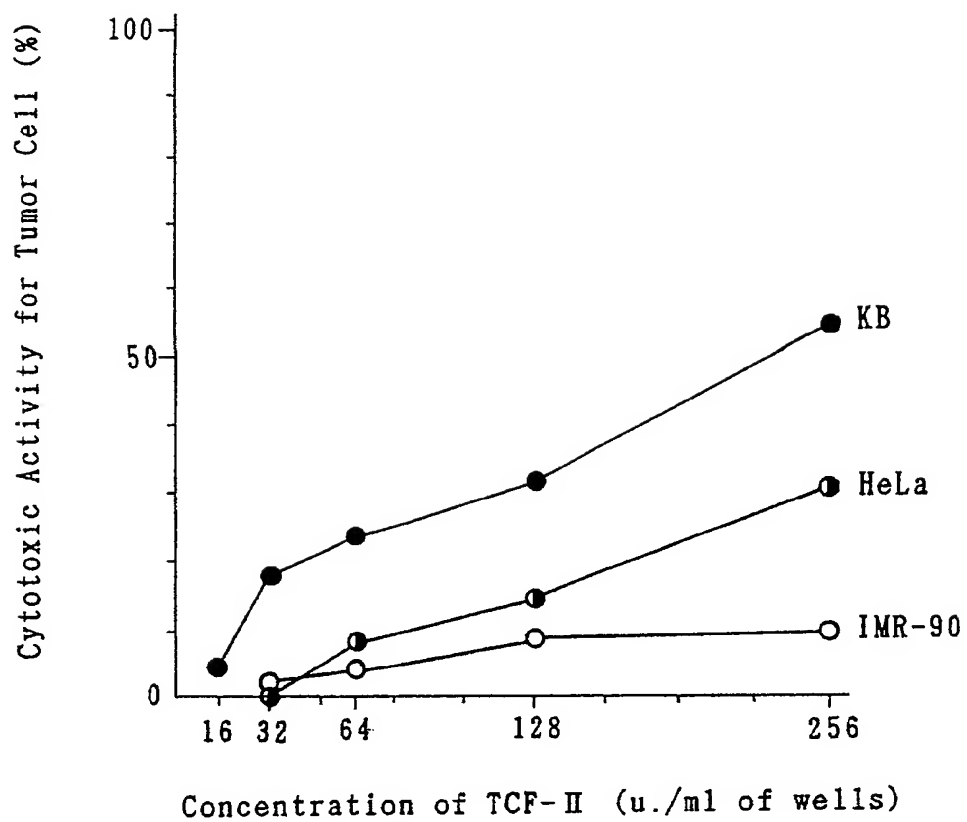
F I G . 6



F I G . 7



F I G . 8



F I G . 9

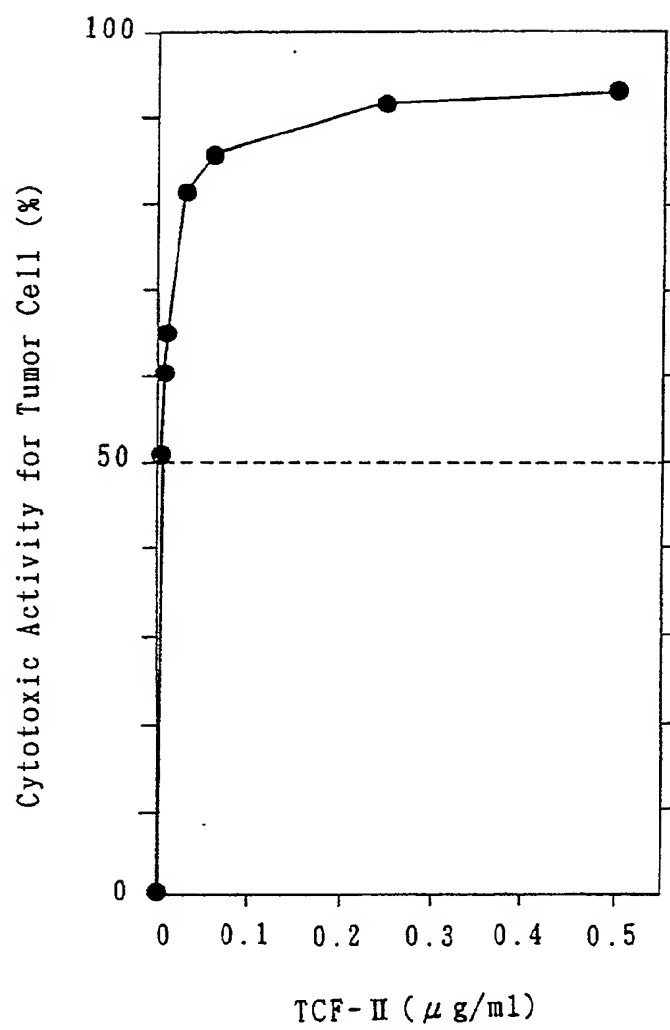
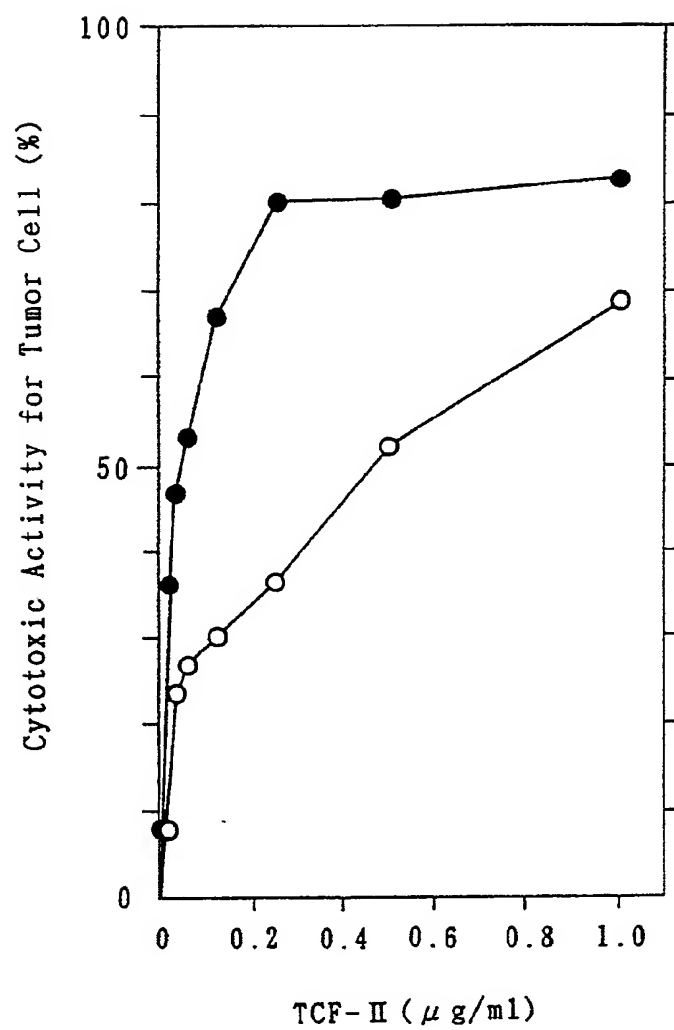
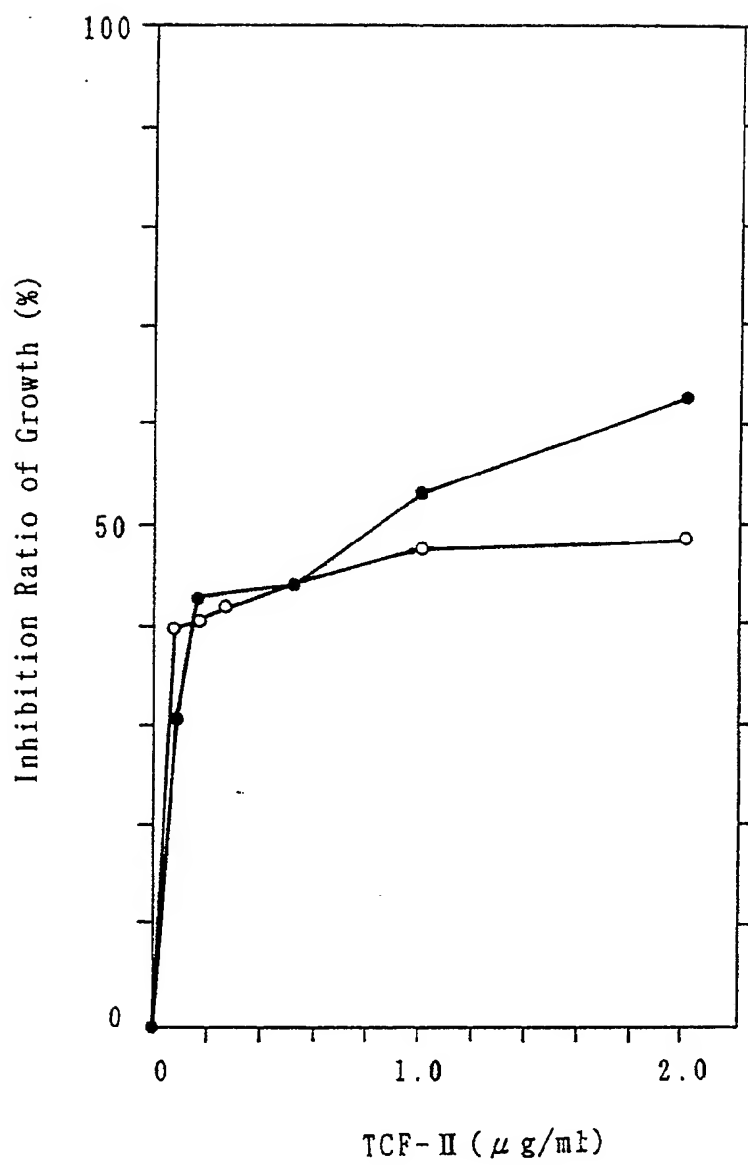


FIG. 10



F I G . 1 1





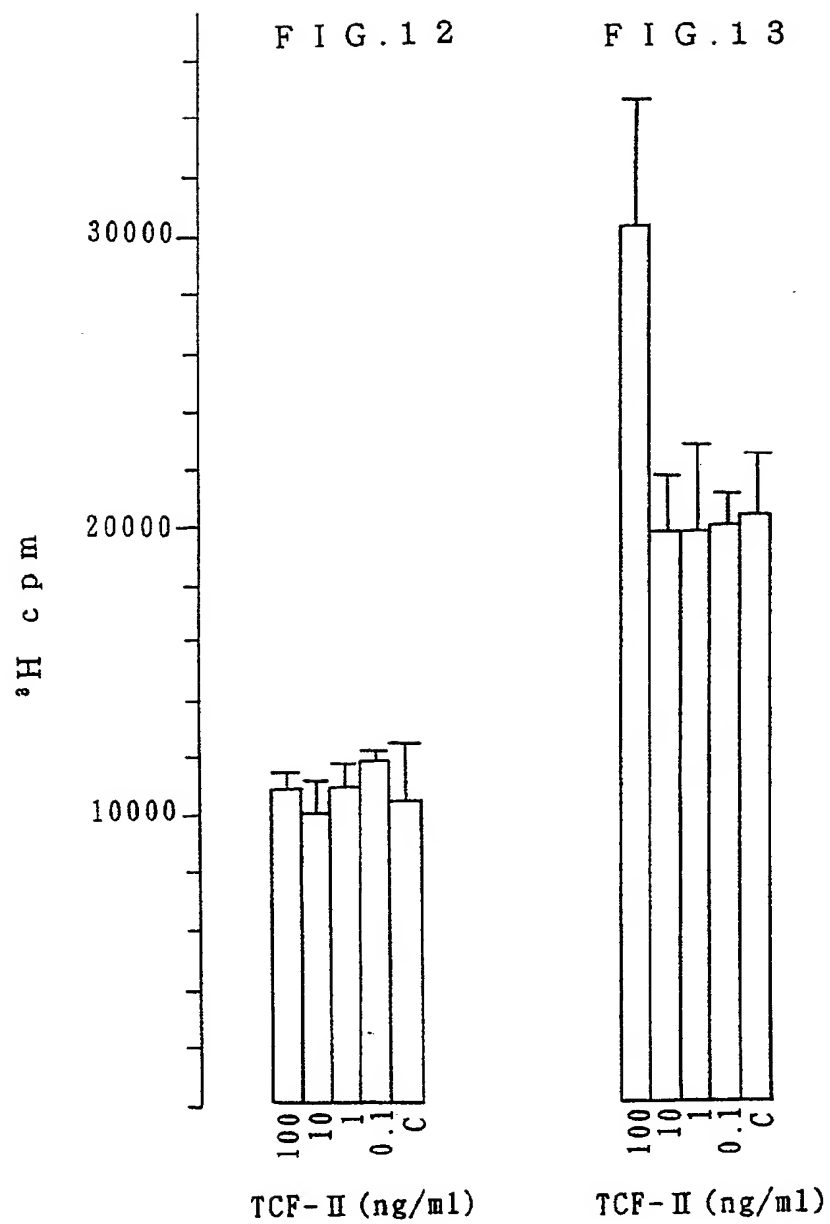


FIG. 14

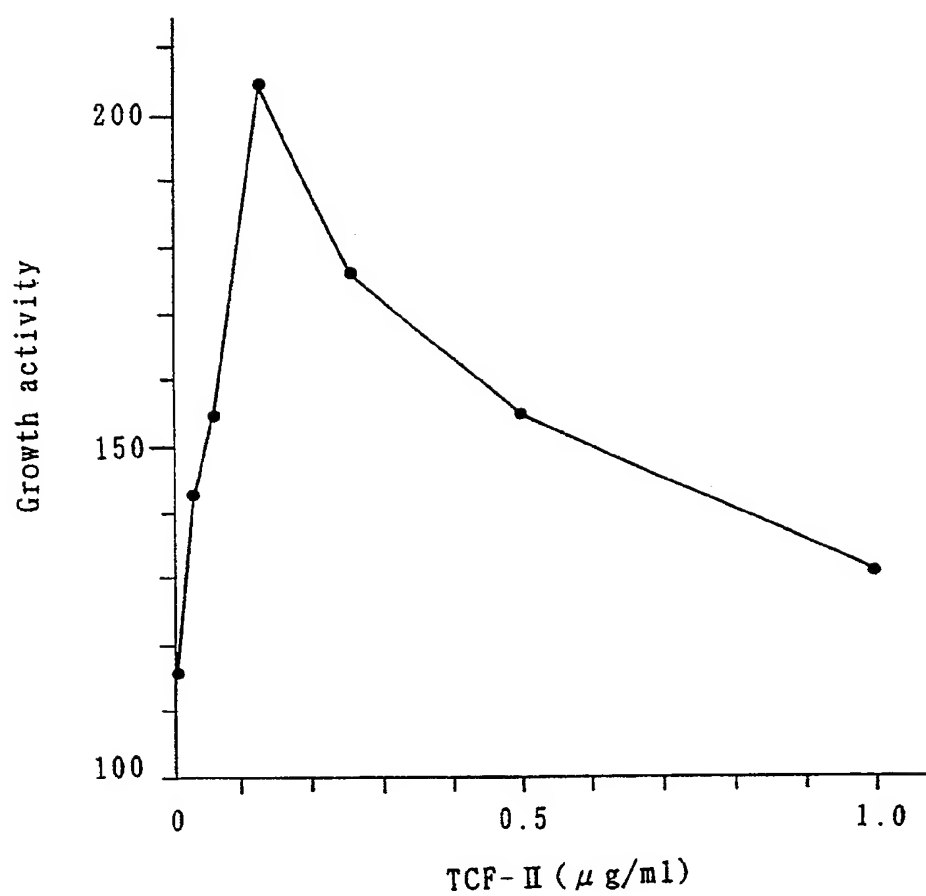


Fig.15 (1)

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-110      -100      -90      -80      -70      -60
TAGGCACTGACTCCGAA

-50      -40      -30      -20      -10      0
CAGGATTCTTTCACCCAGGCATCTCCTCCAGAGGGATCCGCCAGCCCGTCCAGCAGCACC

10      20      30      40      50      60
ATGTGGGTGACCAAACTCCTGCCAGCCCTGCTGCTGCAGCATGTCTCTGTCATCTCCTC
M W V T K L L P A L L L Q H V L L H L L

70      80      90      100      110      120
CTGCTCCCATCGCCATCCCCTATGCAGAGGGACAAAGGAAAAGAAATAACAATTCAT
L L P I A I P Y A E G Q R K R R N T I H

130      140      150      160      170      180
GAATTCAAAAATCAGCAAAGACTACCCTAATCAAAATAGATCCAGCACTGAAGATAAAA
E F K K S A K T T L I K I D P A L K I K

190      200      210      220      230      240
ACCAAAAAAGTGAATACTGCAGACCAATGTGCTAATAGATGTACTAGGAATAAAGGACTT
T K K V N T A D Q C A N R C T R N K G L

250      260      270      280      290      300
CCATTCACTTGCAAGGCTTTTGTGTTTATGATAAGCAAGAAAACAATGCCTCTGGTTCCCC
P F T C K A F V F D K A R K Q C L W F P

310      320      330      340      350      360
TTCAATAGCATGTCAAGTGGAGTGAAAAAGAATTTGCCCATGAATTTGACCTCTATGAA
F N S M S S G V K K E F G H E F D L Y E

370      380      390      400      410      420
AACAAAGACTACATTAGAACTGCATCATTGGTAAAGGACGCAGCTACAAGGGAACAGTA
N K D Y I R N C I I G K G R S Y K G T V

430      440      450      460      470      480
TCTATCACTAAGAGTGGCATCAAATGTCAGCCCTGGAGTTCCATGATACCAACGAAAC
S I T K S G I K C Q P W S S M I P H E H

490      500      510      520      530      540
AGCTATCGGGGTAAAGACCTACAGGAAAACACTACTGTGAAATCCTCGAGGGGAAGAAGGG
S Y R G K D L Q E N Y C R N P R G E E G

550      560      570      580      590      600
GGACCCCTGGTGTTCACAAGCAATCCAGAGGTACGCTACGAAAGTCTGTGACATTCCTCAG
G P W C F T S N P E V R Y E V C D I P Q

610      620      630      640      650      660
TGTTCAAGATTGAATGCATGACCTGCAATGGGGAGAGTTATCGAGGTCTCATGGATCAT
C S E V E C M T C N G E S Y R G L M D H

670      680      690      700      710      720
ACAGAATCAGGCAAGATTGTGACGCGCTGGGATCATCAGACACCACACCGGCACAAATTC
T E S G K I C Q R W D H Q T P H R H K F

730      740      750      760      770      780
TTGCCCTGAAAGATATCCCGACAAGGGCTTTGATGATAATTATTGCCGCAATCCCGATGGC
L P E R Y P D K G F D D N Y C R N P D G

790      800      810      820      830      840
CAGCCGAGGCGCATGGTGTATACTCTTGACCCTCACACCCGCTGGGAGTACTGTGCAATT
Q P R P W C Y T L D P H T R W E Y C A I

850      860      870      880      890      900
AAAAACATGCGCTGACAATATGAATGACACTGATGTTCTTTGGAAACAACCTGAATGC
K T C A D N T M N D T D V P L E T T E C

910      920      930      940      950      960
ATCCAAGGTCAAGGAGAAGGCTACAGGGGCACTGTCAATACCATTGGGAATGGAATTC
I Q G Q G E G Y R G T V N T I W N G I P

970      980      990      1000      1010      1020
TGTCAGCGTTGGGATTCTCAGTATCCTCAGGAGCATGACATGACTCCTGAAAATTTCAAG
C Q R W D S Q Y P H E H D M T P E N F K

1030      1040      1050      1060      1070      1080
TGCAAGGACCTACGAGAAAATTACTGCCGAAATCCAGATGGGTCTGAATCACCTGGTGT
C K D L R E N Y C R N P D G S E S P W C

1090      1100      1110      1120      1130      1140
TTTACCAGTATCCAAACATCCGAGTTGGCTACTGCTCCCAAAATTCCAAACCTGTGATATG
F T T D P N I R V G Y C S Q I P N C D N

```

Fig. 15 (2)

1150 1160 1170 1180 1190 1200  
 TCACATGGACAAGATTGTTATCGTGGGAATGGCAAAATTATATGGGCAACTTATCCCAA  
 S H G Q D C Y R G N G K N Y M G N L S Q  
 internal amino acid sequence  
 1210 1220 1230 1240 1250 1260  
ACAAGATCTGGACTAACATGTTCAATGTGGGACAAGAACATGGAAGACTTACATCGTCAT  
 T R S G L T C S M W D K N M E D L H R H  
 in  $\alpha$ -chain  
 1270 1280 1290 1300 1310 1320  
 ATCTTCTGGGAACAGATGCAAGTAAGCTGAATGAGAATTACTGCCGAAATCCAGATGAT  
 I F W E P D A S K L N E N Y C R N P D D  
 1330 1340 1350 1360 1370 1380  
 GATGCTCATGGACCCTGGTGTACACGGGAATCCACTCATTCCCTTGGGATTATGGCCCT  
 D A H G P Y C Y T G N P L I P W D Y C P  
 1390 1400 1410 1420 1430 1440  
 ATTTCTCGTTGTGAAGGTGATACACACCTACAATAGTCAATTTAGACCATCCCGTAATA  
 I S R C E G D T T P T I Y N L D H P Y I  
 1450 1460 1470 1480 1490 1500  
 TCTTGTGCCAAAACGAACAATTCCGAGTTTGTAAATGGGATTCCAACACGGAACAACATA  
 S C A K T K Q L R Y V N G I P T R T N I  
 N-terminal in  $\beta$  chain N-terminal amino acid sequence  
 1510 1520 1530 1540 1550 1560  
GGAATGGATGTTAGTTGAGATACAGAAATAACATACTCGGAGGATCATTGATAAAG  
 G W M Y S L R Y R N K H I C G G S L I K  
 in  $\beta$ -chain  
 1570 1580 1590 1600 1610 1620  
 GAGAGTTGGGTTCTTACTGCACGACAGTGTTCCTTCTCGAGACTTGAAAGATTATGAA  
 E S W Y L T A R Q C F P S R D L K D Y E  
 1630 1640 1650 1660 1670 1680  
 GCTTGGCTTGGAAATTCATGATGTCCACGGGAAGAGGAGATGAGAAATGCAAACAGGTCTC  
 A W L G I H D V H G R G D E K C K Q Y L  
 1690 1700 1710 1720 1730 1740  
 AATGTTTCCAGCTGGTATATGGCCCTGAAGGATCAGATCTGGTTTAAATGAAGCTTGCC  
 N Y S Q L V Y G P E G S D L Y L M K L A  
 1750 1760 1770 1780 1790 1800  
 AGGCCCTGCTGCTGGATGATTITGTAGTACGATTGATTTACCTAATTATGGATGCACA  
 R P A V L D D F Y S T I D L P N Y G C T  
 1810 1820 1830 1840 1850 1860  
 ATTCCTGAAAAGACCAAGTTGCAGTGTATGGCTGGGGCTACACTGGATTGATCAACTAT  
 I P E K T S C S V Y G W G Y T G L I N Y  
 internal amino acid sequence  
 1870 1880 1890 1900 1910 1920  
GATGGCCTATTACGAGTGGCAGATCTCTATATAATGGGAAATGAGAAATGCAGCCAGCAT  
 D G L L R Y A H L Y I M G N E K C S Q H  
 in  $\beta$ -chain  
 1930 1940 1950 1960 1970 1980  
 CATCGAGGGAAGGTGACTCTGAATGAGTCTGAAATATGCTGCTGGGGCTGAAAAGATTGGA  
 H R G K Y T L N E S E I C A G A E K I G  
 1990 2000 2010 2020 2030 2040  
 TCAGGACCATGTGAGGGGATTATGGTGGCCCACTTGTGTGAGCAACATAAAATGAGA  
 S G P C E G D Y G G P L Y C E Q H K M R  
 2050 2060 2070 2080 2090 2100  
 ATGGTTCTTGGTGTCAATGTTCTGCTGGTGGATGTGCCATTCCAATCGTCTGGTATT  
 M Y L G Y I V P G R G C A I P N R P G I  
 2110 2120 2130 2140 2150 2160  
 TTTGTCCGAGTAGCATATTATGCAAAATGGATACACAAAATTATTTAATCATATAAGGTA  
 F Y R V A Y Y A K W I H K I I L T Y K Y  
 2170 2180 2190 2200 2210  
 CCACAGTCATAGCTGAAGTAAGTGTGTCTGAAGCACCCACCAATACAACCTGT  
 P Q S \*

Fig. 16

```

1
MWVTKLLPALLLQHVLHLLLLPIAIPYAEGQRKRRNTIHEFKKSAKTTLIKIDPALKIK
*****
MWVTKLLPALLLQHVLHLLLLPIAIPYAEGQRKRRNTIHEFKKSAKTTLIKIDPALKIK
1

61
TKKVNTADQCANRCTRNKGLPFTCKAFVFDKARKQCLWFFNSMSSGVKKEFGHEFDLYE
*****
TKKVNTADQCANRCTRNKGLPFTCKAFVFDKARKQCLWFFNSMSSGVKKEFGHEFDLYE
61

121
NKDYIRNCIIGKGRSYKGTVSITKSGIKCQPWSSMIPHEH-----SYRGKDLQENYCRNP
*****
NKDYIRNCIIGKGRSYKGTVSITKSGIKCQPWSSMIPHEHSFLPSSYRGKDLQENYCRNP
121

176
RGEEGGPWCFTSNPEVRYEVCDIQCSEVECMTCNGESYRGLMDHTESGKICQRWDHQTP
*****
RGEEGGPWCFTSNPEVRYEVCDIQCSEVECMTCNGESYRGLMDHTESGKICQRWDHQTP
181

236
HRHKFLPERYPDKGFDDNYCRNPDGQPRPWCYTLDPHTRWEYCAIKTCADNTMNDTDVPL
*****
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241

296
ETTECIQGQGEGRGTVNTIWNIGPCQRWDSQYPHEHDMTPENFKCKDLRENYCRNPDGS
*****
ETTECIQGQGEGRGTVNTIWNIGPCQRWDSQYPHEHDMTPENFKCKDLRENYCRNPDGS
301

356
ESPWCFTTDPNIRVGYCSQIPNCDSHGGDCYRGNKKNYMGNLSQTRSGLTCSMWDKNME
*****
ESPWCFTTDPNIRVGYCSQIPNCDSHGGDCYRGNKKNYMGNLSQTRSGLTCSMWDKNME
361

416
DLHRHIFWEPDASKLNENYCRNPDDAHGPWCYTGNPLIPWDYCPISRCEGDTTPTIVNL
*****
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421

476
DHPVISCAKTKQLRVVNGIPTRTNIGWMVSLRYRNKHI CGGSLIKESWVLTARQCFFSRD
*****
DHPVISCAKTKQLRVVNGIPTRTNIGWMVSLRYRNKHI CGGSLIKESWVLTARQCFFSRD
481

536
LKDYEAWLGIHVDVHGRGDEKCKQVLNVSQVLVYGPEGSDLVLMKLARPAVLDDFVSTIDL
*****
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541

596
NYGCTIPEKTSVYGWGTYTGLINYDGLLRVAHLYIMGNEKCSQHHRGKVTLNESEICAG
*****
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601

656
AEKIGSGPCEGDYGGPLYCEQHKMRMVLGVIVPGRGCAIPNRPGLFVRVAYYAKWIKII
*****
AEKIGSGPCEGDYGGPLYCEQHKMRMVLGVIVPGRGCAIPNRPGLFVRVAYYAKWIKII
661

716      723
LTYKVPQS
"
LTYKVPQS
721      728

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# INTERNATIONAL SEARCH REPORT

International Application No. PCT/JP90/00314

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int. Cl. <sup>5</sup> C07K15/14, A61K37/02, C12N15/19//C12P21/02 (C12P21/02, C12R1:91)		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
IPC	C07K15/14, A61K37/02, C12N15/19, C12P21/00, 21/02	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
Biological Abstracts Data Base (BIOSIS)		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
A	JP, A, 58-148826 (Mochida Pharmaceutical Co., Ltd.), 5 September 1983 (05. 09. 83) & DE, A1, 3306297 & FR, A1, 2522267 & US, A, 4481137	1 - 12
A	JP, A, 1-10998 (The Green Cross Corp.), 13 January 1989 (13. 01. 89) & EP, A2, 322084	1 - 12
<p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"Z" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
May 31, 1990 (31. 05. 90)	June 18, 1990 (18. 06. 90)	
International Searching Authority	Signature of Authorized Officer	
Japanese Patent Office		